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DEVELOPMENT OF A MULTI-PARTICULATE FORMULATION OF VIABLE BACTERIA FOR ORAL AND VAGINAL DELIVERY

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LIST OF BACTERIAL SPECIES

B. lactis	*Bifidobacterium lactis*
E. coli	*Escherichia coli*
G. vaginalis	*Gardnerella vaginalis*
L. acidophilus	*Lactobacillus acidophilus*
L. crispatus	*Lactobacillus crispatus*
L. fermentum	*Lactobacillus fermentum*
L. gasseri	*Lactobacillus gasseri*
L. jensenii	*Lactobacillus jensenii*
L. lactis	*Lactococcus lactis*
L. rhamnosus	*Lactobacillus rhamnosus*

LIST OF ABBREVATIONS

API	active pharmaceutical ingredient
BV	bacterial vaginosis
cfu	colony forming units
DE	dextrose equivalent
dpm	dips per minute
DVS	dynamic vapour sorption
EPS	exopolysaccharide
EVA	ethylene vinyl acetate
GRAS	generally regarded as safe
hIL	human interleukin
HIV	human immunodeficiency virus
HPMC	hydroxypropylmethylcellulose
HPMCAS	hydroxypropylmethylcellulose acetate succinate
IFN	interferon
IL	interleukin
LAB	lactic acid bacteria
MCC	microcrystalline cellulose
MD	maltodextrin
OD	optical density
ON	overnight
PVA	polyvinyl acetate
RH	relative humidity
RSP	riboflavin sodium phosphate

SD	standard deviation
T_g	glass transition temperature
T_0	zero mobility temperature
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TSA	tryptic soy agar
TTFC	tetanus toxin fragment C
UV	ultraviolet

SITUATION AND AIM

Lactic acid bacteria (LAB) comprise a group of Gram-positive bacteria that are characterised by the production of lactic acid as the major end product of their carbohydrate fermentation. LAB are often used for the production and preservation of fermented foods and have as such obtained the ‘generally regarded as safe‘ (GRAS) status which makes them suitable for pharmaceutical applications. Some LAB are also commensals of the human gastro-intestinal or vaginal microflora.

Health promoting effects are attributed to different strains of this group, referred as probiotic strains. Probiotics are defined as living microorganisms which confer a health benefit on the host when supplied in adequate amounts. In various disorders such as gastro-intestinal diseases, bacterial vaginosis and urinary tract infections probiotic strains have been shown to exert a beneficial effect (Naidu et al. 1999, Reid 2001, Reid 2005). In addition to their health promoting effect, LAB have been genetically modified to produce therapeutic proteins or peptides (Hanniffy et al. 2004) to obtain a therapeutic effect after administration.

However, formulation of these microbial strains in a stable formulation containing a high dose of viable bacteria remains challenging due to the detrimental effects of dehydration and of gastric passage on the survival of the bacteria. This project focuses on the development of (a) an oral solid dosage form for the delivery of a genetically modified *Lactococcus lactis* strain and (b) a vaginal delivery system for delivery of different probiotic *Lactobacillus* strains.

The aim of the first part of the thesis was to develop a dry formulation of a recombinant *L. lactis* Thy 12 strain. *L. lactis* Thy 12 was genetically modified in order to produce IL-10, an anti-inflammatory cytokine used in the treatment of Crohn’s disease (Steidler et al. 2003). The main challenges are (a) to design a formulation using a suitable production technique which

preserves the viability of the bacteria since a high number of viable bacteria is required to obtain sufficient levels of therapeutic IL-10 at the target site and (b) to target the genetically modified organisms to the ileal part of the intestine which is the main inflammation site in Crohn's disease patients.

The aim of the second part of the thesis was to develop a suitable vaginal formulation for the efficient delivery of probiotic strains. The evaluated probiotic strains (*Lactobacillus jensenii, Lactobacillus crispatus* and *Lactobacillus gasseri*) were selected based on their characteristics (capacity to produce H_2O_2, inhibition of *Gardnerella vaginalis*, *Atopobium vaginae* and *Escherichia coli*) in relation to the treatment or prevention of bacterial vaginosis (Saerens 2006). The main challenges are (a) to ensure the viability of the lactobacilli during processing (i.e. when dehydrated) and long term storage, (b) to obtain a long vaginal retention time of the dosage forms and (c) to have adequate colonisation of the probiotics in the vaginal cavity. To meet these challenges different dosage forms (layered multi-particulates, freeze-dried powder) were developed and the most suitable formulations/dosage forms were vaginally administered to healthy volunteers to evaluate the retention time, spreading of the formulation and colonisation potential of the selected strains.

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1

INTRODUCTION

1.1. Introduction

This research project focuses on the development of solid dosage forms for the delivery of micro-organisms. More specifically an oral formulation of a genetically modified *Lactococcus lactis* strain was developed in the first part, while the second part aims to develop a vaginal formulation of probiotic *Lactobacillus* strains.

Genetically modified *L. lactis* organisms and their use in disease treatment are discussed in this introductory chapter. Furthermore, an overview is given about the pros and cons of the different vaginal formulations, and the use of *Lactobacillus* strains as probiotic therapy in bacterial vaginosis is described. The stabilisation of bacteria in dry formulations is reviewed, including drying techniques and stabilisation mechanisms. Since multi-particulate oral dosage forms are formulated in this study and fluid bed processing is used for drying and coating of the bacterial formulation, these aspects are also presented in this chapter.

1.2. Genetically modified *Lactococcus lactis*

1.2.1. Crohn's disease

Crohn's disease and colitis ulcerosa are the two most important forms of chronic intestinal inflammations in the Western world (Baumgart and Carding 2007). Crohn's disease mostly affects the entire gastro-intestinal tract, whereas only the colon is inflamed in patients with colitis ulcerosa. The incidence of Crohn's disease has increased in Europe and is mostly diagnosed in young adults. The disease is characterised by a transmural, discontinuous inflammation of the intestinal wall with ulcerations, strictures and fistulae. It predominates in the lower small intestine (ileum) and in the colon. Gut inflammation can lead to malabsorption of vitamins and the frequently occurring gastrointestinal bleedings can result in anaemia. Crohn's disease is also associated with the occurrence of colon cancer.

The etiology still remains partly unknown but it is common that there is an imbalance between pro- and anti-inflammatory modulators in the intestinal wall (Duchmann and Zeitz 1999). Pro-inflammatory interleukins (IL-1, IL-6, IL-8), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) levels are elevated in the gastrointestinal tract of patients with Crohn's disease, as a result of an abnormal T-cell response to commensal microflora in the gut. As in 2.2 – 16.2 % of the cases, patients have a relative member who is also affected, arguments arise that there is a genetic origin. As the incidence of Crohn's disease in the Western world is much higher than in developing countries, environmental factors like food and living conditions also play a role in the pathogenesis of Crohn's disease.

Treatment consists on one hand of acute treatment of the active disease to bring the patient in remission and on the other hand of maintenance treatment to keep a patient in remission (Stites et al. 1994, Baumgart and Sandborn 2007). In patients with a mild form of the disease

aminosalicylates, sulfasalasin and mesalamin are used. In patients with an active inflammation corticosteroids are used, however due to adverse effects and resistance long term use of corticosteroids is not acceptable. Immunosuppressants are suggested as maintenance treatment. Often, surgery is necessary with bowel resection, however this does not induce complete healing as in 80 % of the patients relapses are reported within 1 year.

Recently, biological treatment using cytokines, growth factors, monoclonal antibodies and probiotics has gained interest (Wilhelm et al. 2006; van Deventer 2000). Especially monoclonal antibodies like Infliximab® (which neutralises human tumour necrosis factor alpha) served well to keep patients in remission. Restoration of the intestinal mucosa was observed in patients under Infliximab® treatment.

IL-10 is a cytokine with multiple anti-inflammatory and immunoregulatory activities, including T-cell/macrophage depression and inhibition of pro-inflammatory cytokines and interferon-γ synthesis (van Deventer et al. 1997). The importance of IL-10 in the regulation of mucosal inflammation has been demonstrated in several inflammatory bowel disease animal models. IL-10 deficient mice developed a severe transmural inflammation, which was prevented by administration of IL-10 (Kuhn et al. 1993). In a multi-centre, prospective, randomized, double-blind, placebo-controlled study, Crohn's disease patients were treated for 28 consecutive days with subcutaneous IL-10 in different doses. Endoscopic and clinical improvement (based on the Crohn's Disease Activity Index score) was achieved by IL-10 treatment. However, in high doses a pro-inflammatory effect was found after subcutaneous administration by the production of interferon-γ, which might be circumvented by local delivery to the intestinal mucosa (Tilg et al. 2002).

1.2.2. Use of recombinant L. lactis in human health

L. lactis are Gram-positive non-pathogenic bacteria and are classified as “generally regarded as safe” (GRAS) following their long history of use for the production of fermented milk products. *L. lactis* can be genetically modified to produce antigenic proteins for oral vaccination or therapeutic proteins. Oral vaccination against infectious diseases has gained more interest, as via oral vaccination both mucosal and systemic immune responses can be elicited (Clark et al. 2001). Mucosal immunisation is important as the mucosal surfaces are the portals of entry for many pathogenic agents. Moreover, non-invasive vaccination implies more patient compliance. However, most of the vaccines under development are derived from attenuated pathogenic bacteria, with the associated risk of infection in infants, elderly or immunocompromised individuals. Therefore the use of non-pathogenic, non-invasive bacteria as vehicle for oral vaccination or even as vehicle for therapeutic proteins (e.g. IL-10 in Crohn’s disease treatment) is an attractive concept. Intravenous administration of IL-10 as pure protein results in adverse effects and local delivery by oral administration is difficult as IL-10 is highly acid sensitive. Moreover, the purification of pure IL-10 from its natural source is very compromising as it only occurs in very low amounts (Hanniffy et al. 2004). The effectiveness of *L. lactis* as delivery system for antigens or proteins was demonstrated in several studies (Hannify et al. 2004; Norton et al. 1995).

Robinson et al. (1997) used *L. lactis* for mucosal delivery of a tetanus toxin fragment C (TTFC) via the oral route, resulting in a positive mucosal and systemic antibody response. Mice vaccination with *L. lactis* modified to produce TTFC in combination with IL-2 and IL-6 showed a higher immune response than vaccination with *L. lactis* secreting only TTFC, indicating an adjuvant effect of the secreted IL-2 and IL-6 (Steidler et al. 1998). IL-10 secreting *L. lactis* administered in a dose of 2×10^7 cfu resulted in a 50 % decrease in colitis

in mice treated with sodium dextran sulfate to induce colitis and prevented the onset of colitis in IL-10 deficient mice. The therapeutic effect was due to IL-10 synthesised de novo and not due to residual amounts in the inocula, as these remnants would denature under the acidic conditions of the stomach. The delivered bacteria should be alive, because UV-killed bacteria did not show a clinical effect. Active production of IL-10 was demonstrated in IL-10 deficient mice, as IL-10 was detected in the intestine. Two possible routes were proposed by which IL-10 reaches its target: IL-10 secreted in the lumen can be absorbed by responsive cells or lactococci themselves may be taken up by M-cells (Steidler et al. 2000).

As *L. lactis* itself has a very low innate immunogenicity, sequential oral inoculations can be taken without a significant increase in the antibacterial antibody response (Norton et al. 1994).

1.2.3. Biosafety aspects

The use of genetically modified organisms requires some biosafety aspects to prevent spreading of the modified organisms in the environment or the dissemination of antibiotic selection markers or other genetic modifications to other organisms. To this end, the thymidylate synthase gene (*Thy A*), which is essential for the growth of *L. lactis*, was replaced by the expression cassette for human IL-10. The resulting strain was dependent on thymine or thymidine for growth and survival. As the hIL-10 gene was integrated in the chromosome, dissemination of the genetic modification by lateral gene transfer is minimized and no antibiotic resistance marker is required to guarantee stable inheritance of the transgene. And if the intact *thyA* gene was acquired by homologous recombination, the hIL-10 gene would be removed (Steidler et al. 2003). Moreover, as *L. lactis* lacks any evidence to multiply *in vivo*, no intestinal colonisation can occur.

1.3. Probiotic *Lactobacillus* strains for vaginal delivery

1.3.1. Vaginal formulations

1.3.1.1. Physiology of the vagina

The vagina is a canal extending from the vulva to the cervix (Woolfson et al. 2000, Chien and Lee 2002). The fornix forms the connection between the vagina and the cervix, while the introitus is the part of the vagina close to the vulva, the external part of the female genital organs (Fig. 1). The vagina of an adult woman is 6-7 cm in length and is characterised by an exceptional elasticity. The vaginal wall consists of three layers: the epithelial layer, the muscular coat and the tunica adventia. The thickness of the epithelial layer changes during the menstrual cycle and is also age dependent. The vaginal epithelium is usually considered as a mucosal surface, although it has no goblet cells and no mucin release. The vaginal secretions are a mixture of different components, mainly transudates from the blood vessels and cervical secretions. The vaginal pH fluctuates around 4, due to lactic acid production by *Lactobacillus* strains present in the vaginal microflora. The surface of the vagina is composed of numerous folds increasing the surface area of the vaginal wall. The vagina has a rich blood supply and permeability to a wide range of compounds including peptides and proteins (Richardson and Illum 1992). The hepatic first-pass metabolism is avoided, because blood leaving the vagina enters the peripheral circulation via a rich venous plexus.

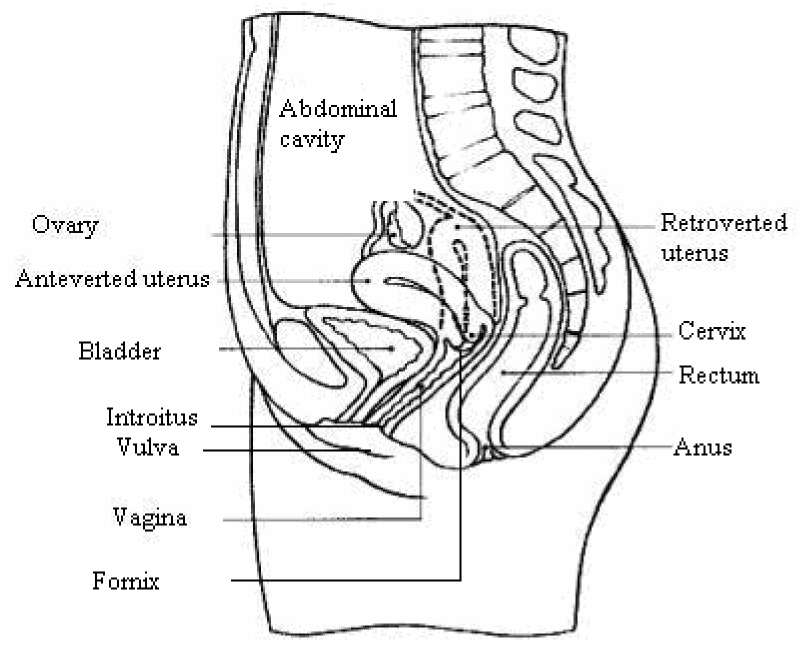

Figure 1 Lateral view of the female pelvis

1.3.1.2. Vaginal formulations on the market

For the development of an acceptable vaginal dosage form some essential requirements are proposed (Hussain and Ahsan 2005; Vermani and Garg 2000):

- easily applicable by the patient
- good spreading leading to an even distribution of the drug
- a sufficiently long retention time to maximise drug release
- no irritation or feeling of dicomfort

Different types of formulations are on the market, each with their advantages and disadvantages. The main group of commercially available vaginal delivery forms are semi-solid dosage forms (creams and gels) used to deliver hormones, antibiotics or fungicides. Gels and creams have been identified as good delivery systems with ease in formulation and administration and a fast drug release. However, these vaginal forms are messy to apply, can

leak in the undergarments and give an uncomfortable feeling to the user (Broumas and Basara 2000; Justin-Temu et al. 2004; Bentley et al. 2000). Moreover, semi-solid formulations may not allow accurate drug dosing due to non-uniform distribution in and leakage from the vaginal cavity. Multiple administrations are required as the retention time is limited (Hussain and Ahsan 2005). Similar problems were reported for pessaries although they are easier to apply and provide accurate drug dosing. Large differences in retention of semi-solid formulations between individuals were reported, varying from 1 to 81 % retained of the initial dose after 24 h (Chatterton et al. 2004). Brown et al. (1997) reported a loss varying from 9 to 97 % two hours post-dosing in post-menopausal women. Barnhart et al. (2005) observed limited vaginal mucosal coverage 24 h after application of a microbicidal vaginal gel. Hydrogels offer prolonged residence due to their swelling and bioadhesive nature leading to controlled release of the drug (das Neves and Bahia 2006).

Vaginal tablets are easy to apply by the user, but vaginal disintegration of conventional tablets can be slow and due to gravity the tablets are rapidly cleared from the vagina. This can be reduced using bioadhesive vaginal tablets, but some studies even reported the loss of bioadhesive tablets after vaginal application (Voorspoels et al. 2002).

Vaginal rings are torus-shaped polymeric devices, most often silicone- or ethylene vinyl acetate-based (EVA), designed to release one or more drugs in a controlled fashion (Alexander et al. 2004). Compared to other vaginal delivery systems, they provide a more accurate and sustained drug dosing, but due to the specific production process not all drugs can be incorporated into vaginal rings.

Most of the vaginal formulations are cleared too fast from the vaginal epithelium as a result of the self cleansing action of the vagina and/or because the vaginal formulation failed to attain a proper distribution over the vaginal mucosa.

1.3.2. Lactobacillus strains

1.3.2.1. Bacterial vaginosis

Bacterial vaginosis (BV) is the most common cause of vaginitis in women of childbearing age (Sobel 2000). A major symptom is an unpleasant smelling, grey-white, thin and homogeneous discharge. Usually there is no pruritus or inflammation and most patients are symptom free. However BV can lead to severe complications, as several studies demonstrated an increased risk of abortion in the first trimester and pre-term birth in women with bacterial vaginosis (Hillier et al. 1995). A significant association exists between the human immunodeficiency virus (HIV) and bacterial vaginosis, suggesting the hypothesis that control of BV can reduce HIV transmission (Schmid et al. 2000).
Diagnosis in clinical practice is based on simple diagnostic criteria proposed by Amsel et al. (1983):

- an adherent homogeneous grey-white vaginal discharge
- “fishy smelling” odour when potassium hydroxide is added to the discharge
- an elevated vaginal pH (> 4.5)
- presence of clue cells, which are vaginal epithelial cells heavily coated with bacteria

BV is characterised by a reduction in the prevalence and concentration of H_2O_2-producing lactobacilli and an increase in the prevalence and concentration of *Gardnerella vaginalis*, *Atopobium vaginae, Mycoplasma hominis*, anaerobic *Peptostreptococcus* species and anaerobic Gram-negative rods belonging to the genera *Prevotella, Porphyromonas* and *Bacteroides* (Fig. 2). The massive overgrowth with vaginal anaerobes is associated with the

production of carboxylase enzymes, which break down vaginal peptides resulting in malodorous components, especially trimethylamine. It is unknown whether the loss of lactobacilli precedes or follows this change in microflora (Sobel 2000).

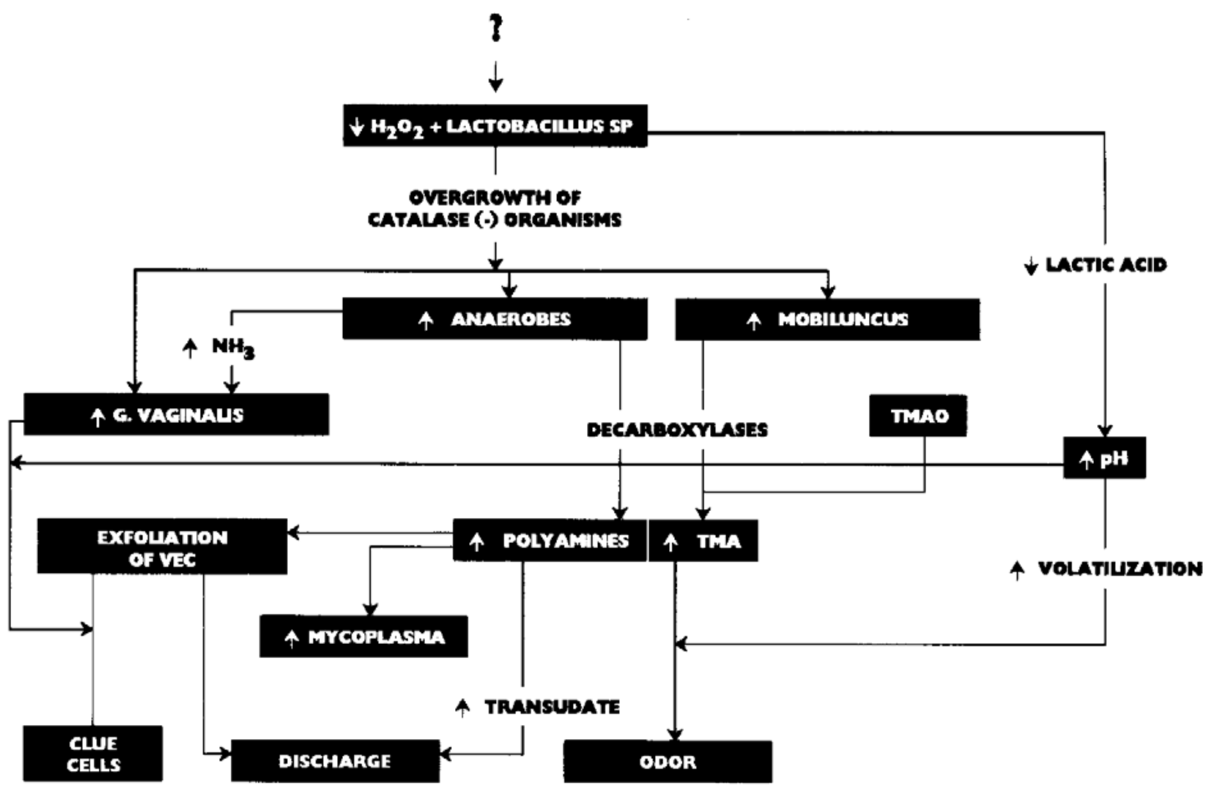

Figure 2 Pathogenesis of bacterial vaginosis

The most successful oral treatment remains metronidazole (500 mg twice daily for 7 days). Topical treatment with clindamycine cream (2 % once daily for 7 days) or metronidazole gel (0.75 % once daily for 5 days) has shown to be as effective as oral metronidazole (Ferris et al. 1995). However the recurrence of BV is very high after antibiotic treatment (30 % after 1 month, 70 % after 3 months). As it is known that lactobacilli can protect against the onset of BV, partly by producing lactic acid to maintain a pH of ± 4, probiotic lactobacilli were suggested as treatment in patients with BV. Also by the release of H_2O_2 or bacteriocins, lactobacilli inhibit the growth of BV-associated microorganisms (Falagas et al. 2007).

Probiotics are defined as living organisms which confer a health benefit to the patient when administered in sufficient amounts.

1.3.2.2. Probiotic strain selection

Bacterial strains suitable as probiotic in the treatment or prevention of BV must fulfil specific requirements (Reid 2001):

- good adherence to the vaginal epithelial cells
- resistant against a large range of bacteriophages
- resistant against a wide variety of bacteriocins
- produce a large amount of lactic acid to maintain a low pH as most bacteria are inhibited by low pH
- produce a large amount of H_2O_2 as hydrogen peroxide has a bactericidal activity against most bacteria
- produce bacteriocins against other species

At the Laboratory for Bacteriology Research (Ghent University Hospital) four strains were selected based on these criteria (Saerens 2006): *Lactobacillus jensenii* PB204-T1-1, *Lactobacillus crispatus* PB125-T1-1, *Lactobacillus crispatus* PB128-T1-1 and *Lactobacillus gasseri* PB088-T2-1. The *L. jensenii* strain is a potent producer of H_2O_2, inhibits the growth of pathogenic bacteria and does not inhibit the growth of *Lactobacillus* species normally present in the vagina. The other strains are also strong inhibitors of *G. vaginalis* and *Atopobium vaginae* and have sufficient H_2O_2 production. A disadvantage of the *L. crispatus* 128 strain is the possible growth inhibition of other *Lactobacillus* strains.

1.3.2.3. Oral versus vaginal delivery

Which administration route is preferable for probiotics remains a matter of debate. In multiple clinical trials both administration routes were used, each with their advantages and disadvantages. Some research groups advocate the oral route for administration. Morelli et al. (2004) demonstrated that oral delivery of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 as freeze-dried preparations induced colonisation of the vaginal microflora. Moreover, Reid et al. (2004) showed in a randomised, placebo-controlled trial that the lactobacilli-dominant microflora was restored in subjects with BV following 2 months of daily oral intake of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 (> 10^9 cfu per dose) and demonstrated an impact on reducing BV occurrences (prevalence of BV fell from 28 to 4 % within the 2-month test period). The mode of action may be multiple, the probiotic strains may ascend from the rectum to colonize the vagina, and/or may immunomodulate the host leading to a reduction of the vaginal sensitivity to BV pathogens (Reid and Bruce 2003). The rectum is assumed as a continuous source for recolonisation of the vagina with bacterial strains (Antonio et al. 2005).

However, the harsh conditions of the gut, including the acidic nature of the stomach and the presence of bile in the intestine, adversely affect the viability of probiotic cultures following oral consumption. So, only a small fraction of orally administered lactobacilli will survive the passage through the gastrointestinal tract and will reach the target viable, which is a requirement to exert beneficial effects. Moreover, the time necessary to reach vaginal colonisation is delayed as several days are necessary for intestinal passage, vaginal ascension and vaginal growth. By vaginal administration of lactobacilli these hurdles are avoided. Although oral ingestion may be considered by most women as an easier way of administration and although intestinal immunomodulatory events may play a role in the probiotic effect,

vaginal administration is a cheaper and more rapid approach to reach high numbers of viable lactobacilli in the vagina. The vaginal instillation of *L. rhamnosus* GR-1 and B-54 or *L. fermentum* RC-14 strains has been shown to reduce the risk of urinary tract infections and to improve the maintenance of a normal microflora (Reid and Burton 2002). Finally, topical administration avoids organoleptic quality considerations and facilitates development and production of the probiotic formulation.

1.4. Stabilisation of bacteria in dry formulations

Preservation of microorganisms by desiccation has been the preferred method for long term storage of cultures. The drying process of a bacterial culture can be summarised as presented in Figure 3. Each step has an influence on the cell recovery after the drying process (Morgan et al. 2006).

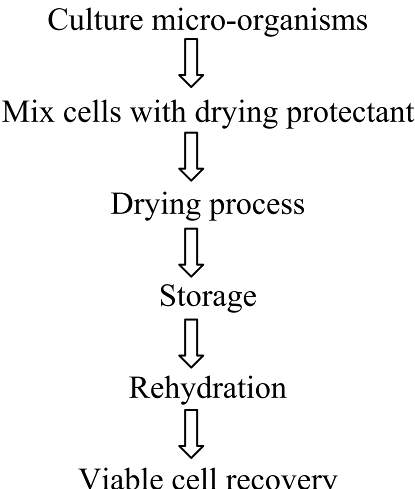

Figure 3 Different steps in the de- and rehydration process of bacterial cultures

1.4.1. Growth conditions

The growth medium itself can have an influence on the survival of bacteria after drying. Carvalho et al. (2004) showed that the type of carbon source added to the growth medium has an influence on the survival ratio after freeze-drying. Bacteria can protect themselves against osmotic stress by accumulating compatible solutes in order to maintain the osmotic balance between the highly concentrated extracellular environment and the more dilute intracellular environment. During a drying process the low water activity can subject the bacteria to osmotic stress conditions. Additives (referred as compatible solutes) like polyols and amino acids can increase the thermotolerance of bacteria (Kets and de Bont 1996).

On the other hand also the growth conditions can influence the survival (Desmond et al. 2002a). If bacteria are grown in stressful conditions (i.e. low pH, high osmotic pressure, high or low temperature) the thermotolerance can increase. A stress response from the microorganisms can result in the production of stress proteins or in physiological changes of the bacterial membrane that increase the tolerance during a drying process (Beales 2004).

1.4.2. External stabilisation

External stabilisation of the culture by the addition of disaccharides, polysaccharides, complex drying media and/or vitamins is necessary to obtain an acceptable viability. Moreover, the survival is strain dependent: while a stabiliser works very well in a specific strain, it can have no beneficial effect during drying of another strain. Two mechanisms of stabilisation are known, i.e. glass formation and water replacement (Aguilera and Karel 1997, Crowe et al. 1998). A combination of both mechanisms is necessary to obtain good stabilisation of bacteria during drying.

1.4.2.1. Glass formation

The external stabilisers should form a glassy matrix during drying. If crystallisation occurs during drying the formed crystal can damage the cell membrane, compromising the integrity of the cell.

A glass is a supersaturated thermodynamically unstable liquid with a high viscosity, which inhibits molecular mobility. Glass formation is characterised by the glass transition temperature (T_g). At a temperature above T_g the amorphous material transfers into a rubbery state with higher mobility, finally resulting in crystallisation and loss of stabilising capacity. At a temperature below T_g the glassy state is maintained. However, for good stabilisation storage at a temperature much lower than T_g is required, referred as T_0 (zero mobility temperature) i.e. at least 50°C below T_g to assure zero mobility (Hatley and Blair 1999).

The higher the molecular weight of the carbohydrates, the higher the T_g. The glass transition temperature is strongly influenced by the water content. By addition of even small amounts of water, T_g can decrease under the storage temperature of the product, resulting in collapse, followed by crystallisation of the product.

1.4.2.2. Water replacement

Bacterial membranes have a lipid bilayer structure in a liquid crystalline state at physiological conditions, in which phospholipids are hydrated with water molecules by hydrogen bonding. During drying, water is removed from the bilayer membranes resulting in a higher packing of the phospholipid head groups, resulting in loss of structural integrity (Fig. 4). Due to Van der Waals interactions the liquid crystalline phase transfers into a gel phase. After rehydration, there is a transition of the gel phase to the liquid crystalline phase, with packing deficiencies

as result and leaking of the intracellular content. However, small hydrophilic molecules (e.g. disaccharides like trehalose) can replace water molecules surrounding the bacterial membrane during drying, thereby maintaining the integrity of the membrane in the absence of water.

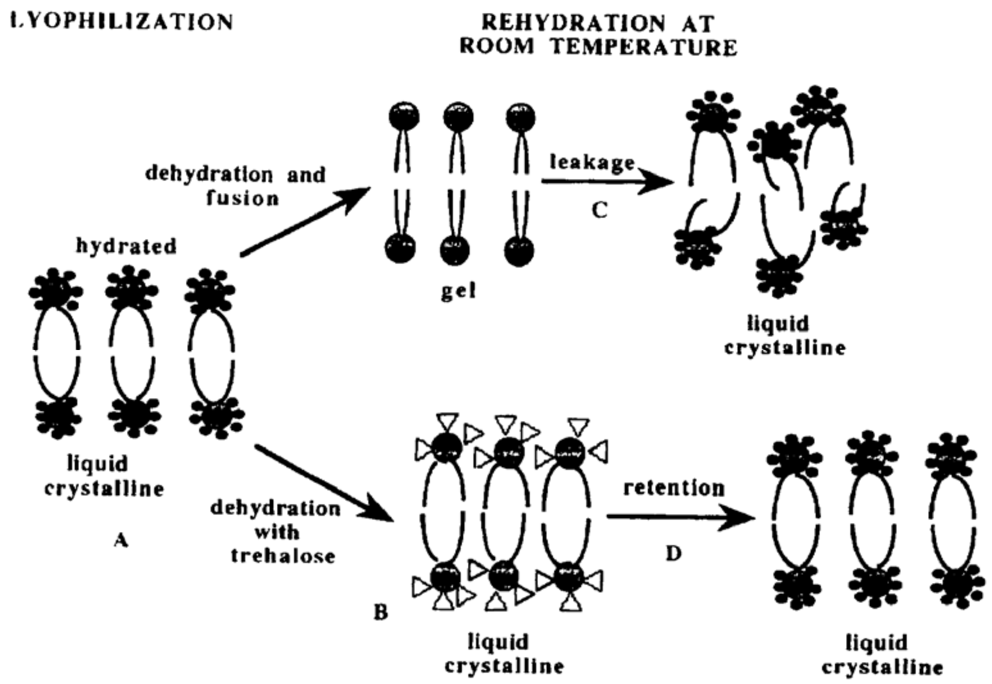

Figure 4 Stabilisation of membranes via water replacement (Crowe et al. 1992)

1.4.2.3. Frequently used stabilisers

Different types of molecules are used as external stabiliser: disaccharides, polysaccharides, complex matrices, vitamins, … Some of these are briefly discussed below, their T_g is presented in Table 1.

1.4.2.3.1. Lactose

Lactose is a disaccharide consisting of glucose and galactose bound via β-1,4 bonds (Fig. 5). Lactose has a relatively high T_g (105°C) and as it is a small molecule it can replace water

molecules during drying and stabilise bacteria via water replacement. Lactose is the main carbohydrate in skim milk, a complex matrix which is also often used to stabilise bacteria. Zarate and Nader-Macias (2006) used lactose in combination with skim milk and ascorbic acid to stabilise probiotic vaginal strains during freeze-drying and storage. For the stabilisation of *Candida sake* Abadias et al. (2001) used different sugars (lactose, trehalose, sucrose), polyols (mannitol, sorbitol) and polymers, but the highest survival after freeze-drying was obtained using a combination of lactose and skim milk. Also *Saccharomyces cerevisiae* was stabilised after freeze-drying by adding lactose to the formulation (Blanquet et al. 2005).

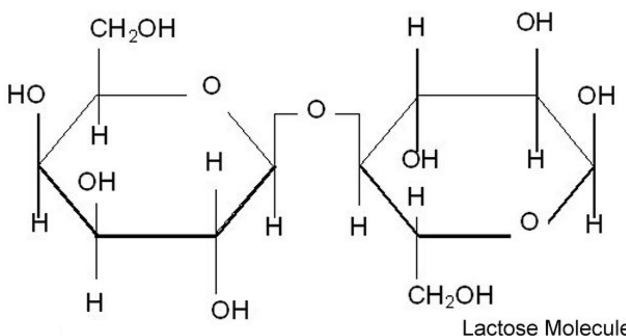

Figure 5 Chemical structure of lactose

1.4.2.3.2. Trehalose

Trehalose is a disaccharide consisting of 2 glucose molecules connected via α-1,1 bonds (Fig. 6). Trehalose has a high T_g (122°C), which is maintained if small amounts of water are added. Small amounts of water result in the conversion of the amorphous trehalose into a crystalline dihydrate form, thereby shielding the remaining glassy trehalose from a decrease in T_g. Because of these characteristics special preservation capacities were attributed to trehalose (Crowe et al. 1996). Leslie et al. (1995) achieved a survival of 69.8 % after freeze-drying of *E. coli* using trehalose as stabilising sugar compared to only 8.1 % without trehalose and 56.2

% in combination with sucrose. However, more recent work stated that trehalose is not more effective than other oligosaccharides. For freeze-drying of *Saccharomyces cerevisiae* no superior survival levels were obtained using trehalose (Blanquet et al. 2005).

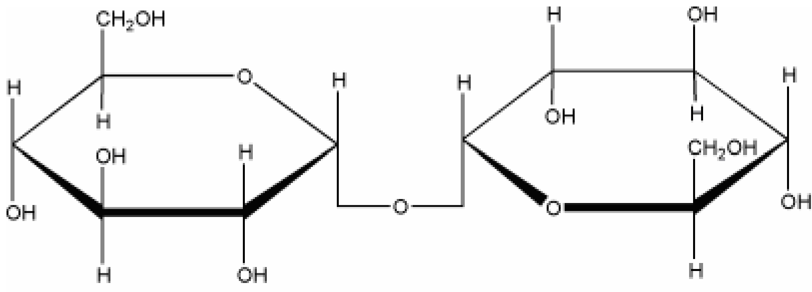

Figure 6 Chemical structure of trehalose

1.4.2.3.3. Maltodextrins

Maltodextrins are hydrolysates of starch, with varying molecular weight (Fig. 7). They are characterised by their dextrose equivalent (DE) value or degree of hydrolysation (eg. a DE value of 38 stands for 38 % hydrolysed starch), hence a high DE value corresponds with a lower molecular weight. High molecular weight maltodextrins have a higher T_g, but replacement of the water molecules surrounding the bacterial bilayer membranes becomes more difficult using maltodextrins with low DE value due to steric hindrance. Andersen et al. (1999) showed that maltodextrins with a low DE value better preserved the acidifying activity of *Streptococcus thermophilus* compared to maltodextrins with a high DE value. Boza et al. (2004) encapsulated *Beijerinckia* sp. in maltodextrins via spray-drying with high survival rates after drying and storage.

Figure 7 Chemical structure of a maltodextrin

1.4.2.3.4. Skim milk

Skim milk is a complex mixture consisting of 91.2 % water, 4.9 % carbohydrates (mainly lactose), 0.1 % fat, 3.1 % proteins (mainly casein) and 0.7 % minerals and vitamins. The stabilising capacity of skim milk during dehydration of bacteria is attributed to its lactose and protein fractions. More than 60 % of *Lactobacillus rhamnosus* GG survived spray-drying when skim milk was used as stabilising matrix (Ananta et al. 2005). Zayed and Roos (2004) combined skim milk with trehalose and sucrose and obtained a survival of about 85 % after freeze-drying of *Lactobacillus salivarus*.

Table 1 T_g of frequently used stabilisers

Stabiliser	T_g (°C)
Lactose	105
Trehalose	122
Maltodextrin DE 5	188
Maltodextrin DE 38	100
Skim milk	72

1.4.3. Drying techniques

The drying technique also has a major influence on the bacterial survival. The most commonly used drying techniques for bacteria are summarised below.

1.4.3.1. Spray-drying

A solution or suspension is sprayed in hot drying air. Via rapid evaporation of the fluid fraction the droplets are dried and the resulting dry powder is collected in a cyclone. Spray-drying is frequently used in the food industry and is a less expensive and faster process compared to freeze-drying. However, due to the high temperatures used in this process (inlet air temperature 190-220°C, outlet air temperature 70-120°C), spray-drying is detrimental for the survival of bacteria (Metwally et al. 1989; Fu and Etzel 1995).

1.4.3.2. Freeze-drying

Freeze-drying is a drying technique where the solvent is frozen and afterwards removed via sublimation (Pikal 1991). Freeze-drying is the most frequently used technique to obtain dried bacterial cultures. The lyophilisation process consists of 3 phases, starting with the freezing step where the temperature of the shelves is lowered and the product is frozen. During this phase a thermodynamically unstable, supercooled solution is formed. Upon ice crystallisation the remaining solutes become concentrated and form an amorphous phase. After the freezing step the frozen water is removed from the samples via sublimation during primary drying at reduced pressure and low temperature. The temperature during primary drying should be sufficiently low to prevent that the water molecules have sufficient mobility to induce product

collapse. This maximum allowed temperature is characterized by the collapse temperature and depends on the formulation. During secondary drying residual (bound) water is removed at reduced pressure and higher shelf temperature. As a hygroscopic powder is obtained, further handling requires some precautions to assure the quality of the freeze-dried product. Freeze-drying is a gentle drying process as drying occurs at low temperature, resulting in a high survival rate of heat sensitive products like proteins and bacteria (Champagne et al. 1991). The most detrimental step is the freezing step and the rate of cooling as the formation of large ice crystals can damage fragile cell membranes. As slow cooling yields larger ice crystals this problem can be avoided by flash freezing in liquid nitrogen (Morgan et al. 2006). A drawback of freeze-drying is the high energy cost and the long duration of the entire process.

1.4.3.3. Layering

Layering is a technique where a solution or suspension is sprayed and subsequently dried on inert carriers using a Wurster-based fluidized bed system (Huyghebaert et al. 2005b). Fluidized bed layering also occurs at elevated temperature, but is much lower (on average about 45°C) than the temperature used during spray-drying. Moreover, this technique results in a multi-particulate formulation, whereas spray- and freeze-drying result in a powder that must be further processed into a pharmaceutical formulation suitable for oral delivery. A multi-particulate pellet formulation assures a fast gastric emptying as it behaves like a liquid when it passes through the gastro-intestinal tract (Krämer and Blume 1994). In case of oral delivery this is an advantage for gastrosensitive products (such as bacteria) to minimize the contact time between gastric acid and layered bacteria. Moreover, the multi-particulate formulation can be enterically coated in the same equipment to protect the product against the gastric environment.

1.4.4. Overview of dried formulations of Lactococcus lactis, Lactobacillus jensenii, Lactobacillus gasseri and Lactobacillus crispatus

1.4.4.1. *Lactococcus lactis*

L. lactis is frequently used in the dairy industry as starter culture. In literature freeze- and spray-drying are used in combination with different protective media to obtain dry starter cultures for the production of dairy products.

Broadbent and Lin (1999) used freeze-drying and observed an increase of survival rate after drying if *L. lactis* ssp. *cremoris* underwent a 2 h cold shock at 10°C prior to drying: 23 ± 6 % survival without cold shock vs. 49 ± 2 % after cold shock. The improved resistance to drying was attributed to the presence of cold shock proteins. Similarly Ziadi et al. (2005) showed an improvement in survival of *L. lactis* ssp. *lactis* after heat treatment for 20 min at 45°C: a survival-rate after freeze-drying of 38.8 % for the heat-treated bacteria vs. 21.9 % without heat-treatment. To and Etzel (1997) obtained a high survival (63 ± 6 %) after freeze-drying of *L. lactis* ssp. *cremoris* using a combination of maltodextrins, lactose and phosphate buffer. Champagne et al. (1996) used reconstituted skim milk supplemented with gelatin, xanthan gum or maltodextrins to improve resistance to freeze-drying of *L. lactis* ssp. *lactis*, however no significant improvement was shown. Carcoba and Rodriguez (2000) also used reconstituted skimmed milk supplemented with different components to improve viability after freeze-drying of *L. lactis* ssp. *lactis* with the highest viability using β-alanine as supplement: a 44.3 % viability using reconstituted skim milk, but 60.7 % if β-alanine was added. Doleyres et al. (2004) cultured *L. lactis* ssp. *lactis* as an immobilised cell culture using κ-carrageenan/locust bean gum gel beads and evaluated the resistance to freeze-drying, however no significant improvement in viability compared to a free cell culture was observed:

39 ± 16 % and 21 ± 11 %, respectively. Looijestijn et al. (2001) compared the survival after freeze-drying of an exopolysaccharide-producing *L. lactis* ssp. *cremoris* with a control strain, resulting in viabilities of 30.3 and 32.2 %, respectively, indicating that exopolysaccharide production did not result in a higher resistance to freeze-drying. Huyghebaert et al. (2005a) obtained a viability of 60 ± 18 % after freeze-drying of *L. lactis* ssp. *cremoris* using skim milk supplemented with glucose and casein hydrolysate.

Vacuum desiccation of *L. lactis* showed the highest survival ratio if mannitol was used as stabiliser as no loss of viability was reported (Efiuvwevwere et al. 1999). Fu and Etzel (1995) used spray-drying for the production of dairy starter cultures of *L. lactis* ssp. *lactis* with the highest survival percentage of 78 ± 12 % if a high initial cell load (1.1×10^{11} cfu/ml) and a low outlet-air temperature of 77°C was used. For *L. lactis* ssp. *cremoris* a survival of only 2.95 ± 0.07 % was obtained using an outlet-air temperature of 65°C (To and Etzel 1997).

Huyghebaert et al. (2005b) used layering to dry *L. lactis* on inert carriers obtaining the highest viability (9 %) using a combination of skim milk and inulin as stabilising matrix.

1.4.4.2. Vaginal probiotic strains: *Lactobacillus jensenii, Lactobacillus gasseri and Lactobacillus crispatus*

Maggi et al. (1994) freeze-dried *L. gasseri* using a combination of skimmed milk and malt extract as stabilising matrix, yielding in a count of 2.0×10^{10} cfu/g. However, viability progressively decreased during storage at 4°C (Maggi et al. 2000). Using the same stabilising matrix Mastromarino et al. (2002) obtained viabilities for *L. gasseri* varying between 9.7 and 10.7 log cfu/g. Otero et al. (2007) reported a similar viability of this probiotic strain using a different matrix (combination of skim milk with sucrose or lactose).

Freeze-drying of *L. crispatus* using skimmed milk and malt extract as stabilising matrix resulted in a viability of 7.0×10^7 cfu/g after 1 month storage at 4°C (Maggi et al. 2000), whereas Mastromarino et al. (2002) reported a viability of 3.3×10^8 cfu/g immediately after freeze-drying using the same stabilisers.

No literature reports were found about the survival of *L. jensenii* after drying.

1.5. Gastric protection of orally delivered bacteria

To reach the gastro-intestinal tract bacteria must pass through the detrimental gastric environment. To assure that a sufficient dose of bacteria reaches the target site, the formulation must be protected against the acidic conditions of the stomach and the bile present in the duodenum, indicating that not only viability after drying is important but also survival after gastric passage of orally delivered bacterial strains. Klijn et al. (1995) reported that only 1 % of *Lactococcus lactis* survived the human gastro-intestinal tract. In literature different approaches are used to obtain enteric protection of a viable bacterial formulation. Favaro-Trindade and Grosso (2002) encapsulated *L. acidophilus* and *B. lactis* by spray-drying using cellulose acetate phthalate as encapsulating agent. Viability of encapsulated bacteria decreased with 1 log unit after 2 h at pH 1, whereas non-encapsulated bacteria were completely destroyed after 1 h. Desmond et al. (2002b) used acacia gum and skim milk as encapsulating agents during spray-drying of *Lactobacillus paracasei,* with a reduction of 4 log units after 2 h in porcine gastric juice at pH 3.0. Stadler and Viernstein (2003) prepared tablets containing lyophilized *L. acidophilus* using hydroxypropylmethylcellulose acetate succinate (HPMCAS) as matrix forming component. The best result was obtained if a combination of HPMCAS and sodium alginate was used, with only 1 log decrease after 2 h in 0.04 N HCl. At higher HPMCAS concentration gastric protection improved, however

disintegration of these tablets was slow when reaching the target site. Calinescu et al. (2005) used carboxymethyl high amylose starch as excipient for *Escherichia coli* oral formulations resulting in non-significant differences in viability before and after 2 h incubation in simulated gastric fluid. In this case also disintegration problems in simulated intestinal fluid were reported. Microencapsulation in calcium alginate beads has been frequently used to protect the strains against the gastric environment by creating a physical barrier. Chandramouli et al. (2004) reported a 2 log decrease in viability of encapsulated *Lactobacillus acidophilus* after 3 h incubation in simulated gastric conditions (pH 2). Co-encapsulation of prebiotics (high amylose corn starch, inulin, oligofructose) within alginate beads and coating of these calcium alginate capsules with chitosan or poly-L-lysine improved protection (Iyer and Kailasapathy 2005, Krasaekoopt et al. 2004). Microencapsulation in a casein/pectin complex did not protect at pH values similar to those of the human stomach (Oliveira et al. 2007). Encapsulation of bacteria in a whey protein-based microcapsule did improve survival in simulated gastrointestinal conditions (Reid et al. 2005, Picot and Lacroix 2004).

This brief summary of literature data about enteric protection of bacteria indicates that it is difficult to sufficiently protect the tested strains during gastric passage.

1.6. Pellets

Pellets are spherical or nearly spherical, free flowing particles with a narrow size distribution, varying between 500 and 1500 µm for pharmaceutical applications (Ghebre-Sellassie and Knoch 2002). Often pellets are produced by extrusion/spheronisation, a multi-step process. In the first step the different components (excipients and/or API) are dry mixed. This powder mix is agglomerated using a granulation liquid, mainly water. The wet mass is extruded

through an extrusion screen having die perforations with the desired diameter. The obtained cylindrical extrudates are broken into cylindrical rods and rounded into spheres by means of a fast-rotating friction plate (Fig. 8). Finally, the pellets are dried, mostly using oven or fluid bed drying.

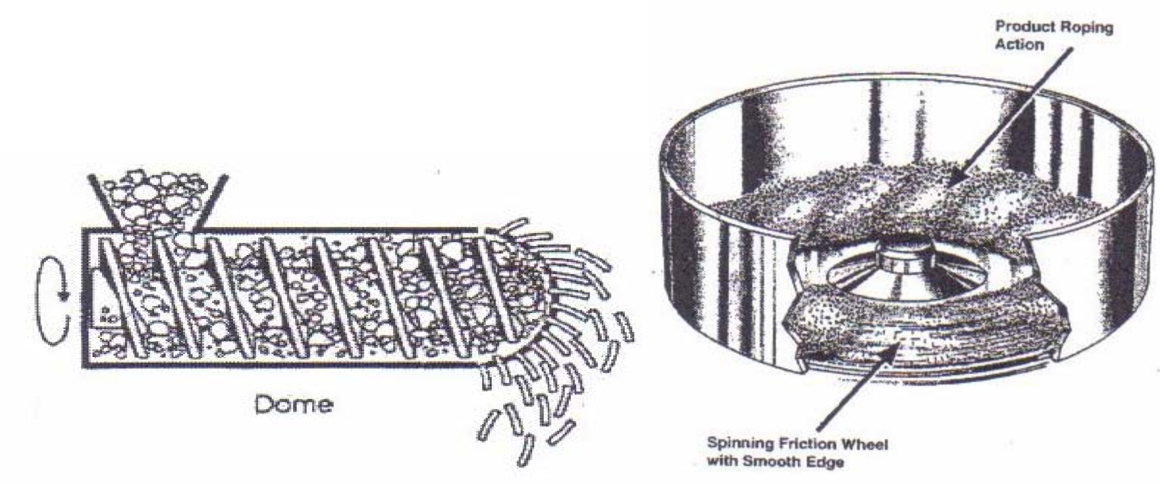

Figure 8 Dome-extruder and spheroniser used for the production of pellets via extrusion/spheronisation

Microcrystalline cellulose (MCC) is commonly used as the main excipient in extrusion/spheronisation. Since microcrystalline cellulose pellets do not disintegrate in aqueous media and drug release occurs via diffusion through an insoluble inert matrix, fast disintegrating pellets were developed by Dukic et al. (2007) using a specific starch grade (UNI-PURE® EX) as main excipient. Pellets are generally used for oral drug delivery of API's, as multi-particulates offer some important pharmacological and technical advantages: fast gastric emptying, decreased risk of dose-dumping in case of coated pellets, narrow size distribution, free flowing, low friability, ...

1.7. Coating/layering of multi-particulate systems

For coating of multi-particulate dosage forms, the Wurster-based fluidized bed system is mainly used. The Wurster-based fluidized bed equipment consists of 4 different regions (Fig. 9) (Christensen and Bertelsen 1997): upbed region, expansion chamber, downbed region and horizontal transport region.

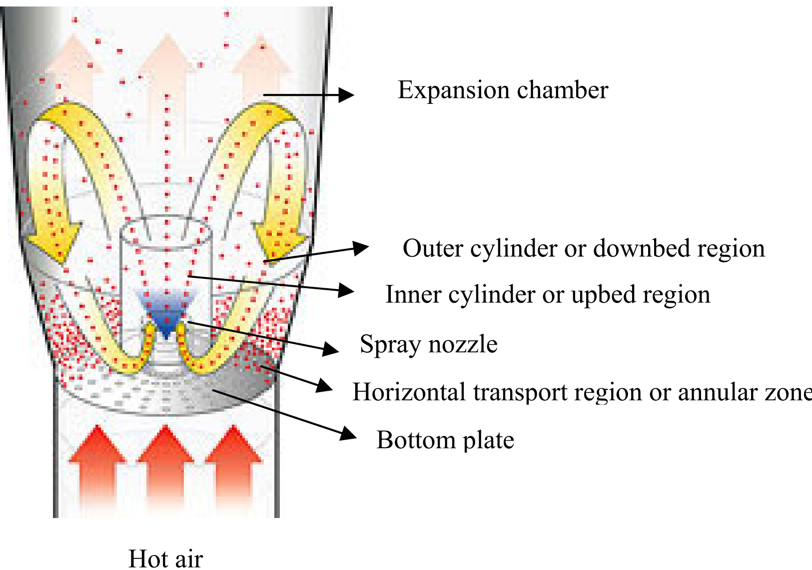

Figure 9 Wurster based fluidized bed system

The coating process is a three-step process with a start-up phase, coating phase and drying (or cooling) phase. During start-up the equipment and circulating product is preheated to the desired coating temperature. Preheating prevents over wetting of the product during the initial application of the coating solution. Heating also facilitates film formation. During the coating phase different processes occur simultaneously. The coating or layering solution is atomised through the spray nozzle to form drops with an average diameter of 10-30 µm. These droplets are transported to, adhere and dry on the substrate. This process is repeated for each layer applied on the product. The solvent evaporation should be controlled in such a way that it is

sufficiently slow to allow dissolution of the first layers to assure a good adhesion of the subsequent layers. However, evaporation should not take too long to avoid particle agglomeration. After coating, there is a terminal drying or cooling step.

When using the Wurster setup in a fluid bed, particles make a controlled movement within the fluid bed to ensure a homogeneous coating layer on the pellets. A fountain movement is created in the fluidisation chamber due to the inner cylindrical tube (upbed region) surrounding the spray nozzle and the specific design of the bottom plate (having a higher number of perforations in the centre). As a result the air velocity in the inner cylinder is higher and particles are transported upwards via the inner cylinder. Simultaneously these particles are coated via droplets atomized through the centrally positioned nozzle. In the expansion chamber the air velocity drops and particles return via the downbed region where the air velocity is lower than the minimum velocity of fluidisation. Clustering of particles occurs most frequently in this region. Via the horizontal transport zone product is transported in the direction of the upbed region.

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2

DEVELOPMENT OF AN ORAL MULTI-PARTICULATE FORMULATION OF VIABLE RECOMBINANT *LACTOCOCCUS LACTIS*

2.1. Introduction

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 (Huyghebaert et al. 2005). 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 (Van Deventer et al. 1997) and which has already been used in an *in vivo* study (Braat et al. 2006). 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 optimisation of several parameters. The influence of process parameters (atomising pressure and nozzle diameter) and carrier material (microcrystalline cellulose pellets or starch-based pellets) on the viability of *L. lactis* Thy 12 during layering was evaluated. Different stabilising

matrices (skim milk, disaccharides, polysaccharides and polyalcohols) were tested for their stabilising capacity 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. Microencapsulation of bacteria, using sodium alginate as encapsulation matrix (Chandramouli et al. 2004), was used as alternative technique to obtain a multi-particulate formulation with a high number of viable bacteria. Before the experiments the Bioscreen turbidity method, used for the viability assessment, was validated.

2.2. Materials and methods

2.2.1. Materials

Growth media M17 (Difco) and GM17T (i.e. M17 supplemented with 0.5 % glucose and 50µg/ml thymidine) were purchased from Becton Dickinson (MA, USA). Different stabilising matrices were used: skim milk (Difco, Becton Dickinson, MA, USA), lactose (α-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), glucose (Bufa, Uitgeest, The Netherlands), poly vinyl alcohol (PVA) (80 and 87 % hydrolyzed) (Sigma-Aldrich, St. Louis, USA), vitamin C (Certa, Braine-l'Alleud, Belgium) and chondroitin sulphate (Sigma-Aldrich, St. Louis, USA). Microcrystalline cellulose spheres (Cellets®, Pharmatrans-Sanaq, Basel, Switserland) and starch-based pellets were used as inert carriers for the layering experiments. Starch-based 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, New Jersey, USA). Sodium alginate (Sigma-Aldrich, Steinheim, Germany) and calciumchloride (Merck, Darmstadt, Germany) were used for the microencapsulation process.

2.2.2. Bacterial strain

L. lactis Thy 12 (human IL-10 producing *L. lactis* subsp. *cremoris* 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. *L. lactis* Thy 12 was genetically modified in order to produce human IL-10 and was made thymidine dependent to avoid spreading of the bacteria in the environment (Steidler et al. 2003).
The bacteria were stored at -20°C as a stock suspension in glycerol/GM17T (50/50).

2.2.3. Validation of the viability assessment method using the Bioscreen turbidity method

Growth media were sterilised using a simple stovetop autoclave (Presto® pressure canner 23 Quart, Eau Claire, Wisconsin, US). Sterilised growth media were stored at 4°C for maximum 1 week before use. The growth media were acclimatised to room temperature before each experiment. Before the onset of a new experiment membrane-sterilised glucose and thymidine were added to the growth medium.
Viability of the bacteria after processing was determined by monitoring the growth of standards and samples in a Bioscreen-C (Labsystem, Helsinki, Finland) via optical density

measurements. Different dilutions (13 in total) of the overnight culture were prepared, using fresh GM17T as diluents and loaded in triplicate into microtiter plates for analysis in the Bioscreen. The growth at 30°C was monitored over 24 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 (Fig. 1). The 50 % time was plotted against the natural logarithm of the viability to calculate the equation of the standard curve (Fig. 2). The viability of a sample was determined based on the standard curve of the overnight culture and expressed as percent survival of the overnight culture. For each drying experiment a new calibration curve was made, as the number of bacteria obtained in the stationary phase was not identical for each culture.

To evaluate the effect of sample preparation on viability, layered pellets were vortexed for 10, 30 or 60 s in 1 or 10 ml sterilised water to release the bacteria from the matrix. Three dilutions of each sample were loaded in duplicate into microtiter plates for analysis in the Bioscreen.

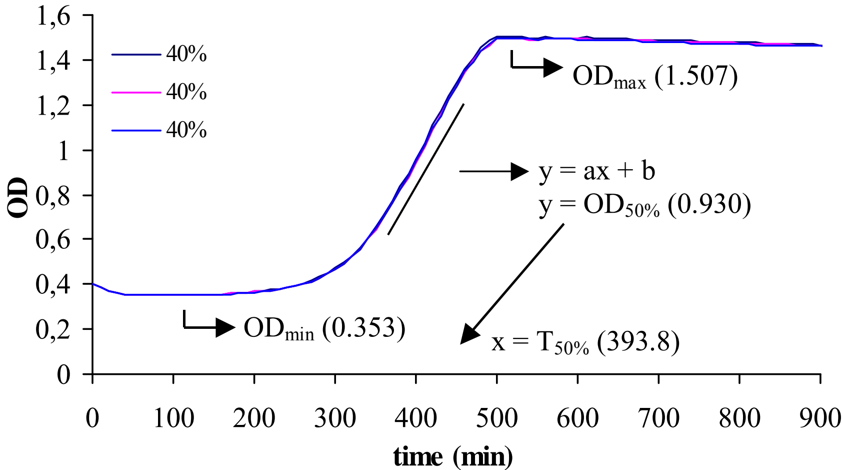


Figure 1 Example of growth curves of 40% dilutions of the starting culture and visualisation of the method used to determine $T_{50\%}$

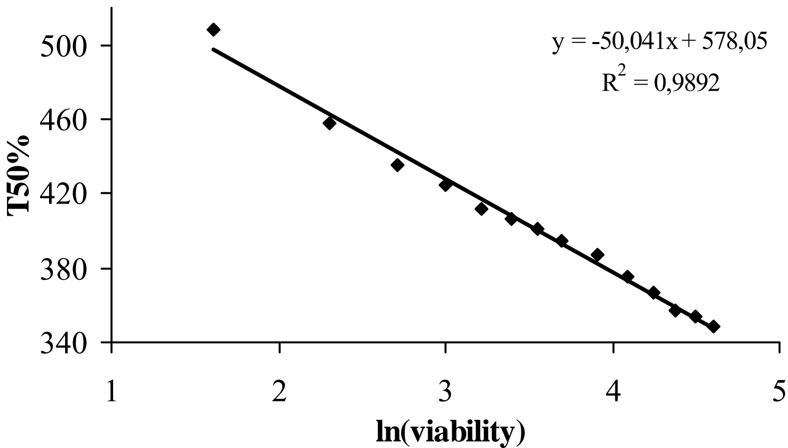


Figure 2 Example of a standard curve ($T_{50\%}$ against the natural logarithm of the viability)

To evaluate the day-to-day variability of the method pellets layered with *L. lactis* were analysed on 6 different days using a single batch of growth medium. To evaluate the influence of the sterilising process on the viability, growth medium autoclaved for 15 (standard process time) or 60 min was used during analysis of a sample via the Bioscreen method.

2.2.4. Preparation of the layering suspensions

L. 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 matrices (Table 1).

To prevent further activity or growth, the cultures were kept on ice in between all handling steps.

Table 1 Overview of different layering matrices

Stabilising matrix		Concentration (w/v)
Influence of stabilising matrix		
Skim milk	Skim milk and components	10 %
Lactose	Skim milk and components	5 %
Ca-caseinate	Skim milk and components	3.8 %
Lactose/Ca-caseinate		5/3.8 %
Trehalose	Disaccharides	5 %
Sucrose	Disaccharides	5 %
Isomalt	Disaccharides	5 %
Mannitol	Polyalcohol	5 %
Maltodextrin DE 5	Polysaccharides	5 %
Maltodextrin DE 18	Polysaccharides	5 %
Maltodextrin DE 38	Polysaccharides	5 %
Glucose		5 %
PVA (80% hydrolyzed)	Synthetic polymers	5 %
PVA (87% hydrolyzed)	Synthetic polymers	5 %
Lactose/vitamin C		5 %/1.25 %
Chondroitin sulphate		5 %
Influence of stabiliser/bacteria ratio		
Skim milk		5 % - 10 % - 20 %
Lactose		2.5 % - 5 % - 10 %
Trehalose		2.5 % - 5 % - 10 %
Longer process time		
Lactose		5 %
Lactose/maltodextrin DE 5		5/2.5 % - 5/5 %
Lactose/skim milk		5/10 %
Higher bacterial cell concentration in layering suspension		
Lactose/skim milk		5/10 % - 10/20 %

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

A layering suspension consisting of *L. lactis* Thy12 (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 using the Bioscreen turbidity method.

2.2.6. Layering process (n = 3)

Microcrystalline cellulose spheres (Cellets®) and starch-based 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-based pellets (900-1400 µm) were produced by extrusion/spheronisation (Dukic et al. 2007). 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 (containing bacteria and stabilising matrix) was kept on ice. 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°C 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 an incubation time of 30, 60, 90 and 120 min.

2.2.6.1. Evaluation of the pellets

Viability of the bacteria after layering was determined by the Bioscreen turbidity method (paragraph 2.2.3). 0.1 g of the layered pellets was vortexed for 30 s in 1 ml sterile water to release the bacteria from the layered pellets. Some samples were evaluated for viability using the plate count method. Via the pour plate 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.

2.2.7. Dynamic vapour sorption

Water sorption isotherms of the layered pellets were determined gravimetrically at 25 ± 0.1°C using a DVS Advantage 1 with a Cahn D200 microbalance (Surface Measurement Systems, London, UK). Between 10 and 20 mg pellets were placed in the sample cup of the instrument, subjected to a drying step in order to bring the sample to a constant weight and subsequently exposed to increasing relative humidity (RH) (using 10 % increments up to 90 % RH). Each step continued until equilibrium was reached (i.e. when the change of mass was smaller than 0.002 % per minute during at least 10 min). The mass change was recorded every minute with a resolution of ± 0.1 µg.

2.2.8. Microencapsulation process

Bacteria of an overnight culture were collected by centrifugation and resuspended in a 2 % sodium alginate solution, enriched with 5 % lactose or 5 % trehalose. Microencapsulation of the bacteria was performed using syringe and needle (diameter: 1.1 mm) via drop wise addition of the sodium alginate/bacteria suspension to a 0.1 M calcium chloride solution, which was continuously stirred using a magnetic stirrer. The capsules were allowed to stand for about 10 min for hardening and were then collected by filtration. The capsules were washed with distilled water. The obtained microcapsules were freeze-dried using an Amsco Finn Aqua GT4 freeze-dryer (Amsco, Germany). The capsules were flash frozen to -70°C via immersion during 1 min in liquid nitrogen or frozen to -45°C within 175 min (by placing the samples on the shelf of the freeze-drier). Primary drying was performed at -15°C and a pressure varying between 0.8 and 1 mbar during 13 h, followed by secondary drying at elevated temperature (10°C) and reduced pressure (0.1–0.2 mbar) for 7 h. In one experiment a 48 h freeze-drying process was used.

2.2.8.1. Evaluation of the microcapsules

Microcapsules were evaluated immediately after production, after the liquid nitrogen freezing step and after freeze-drying. 100 mg microcapsules were stirred for 30 min in 0.1 M sodium citrate solution to release the bacteria from the microcapsules. After 30 min samples were collected and analysed by the Bioscreen turbidity method or the plate count method.

2.2.9. Statistical analysis

The viability values were statistically evaluated with a one-way ANOVA or an independent sample T-test 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 significance level. All analyses were performed with SPSS 12.0 for Windows.

2.3. Results and discussion

2.3.1. Validation of the Bioscreen turbidity method

To evaluate the survival of bacteria after drying an appropriate viability measurement technique should be used. Although different methods are available for the viability assessment of bacteria (Caldwell 1995), the most commonly used technique is the plate count method. A quantitative dilution of a bacterial suspension is prepared and distributed within or on the surface of an agar plate and incubated for 1-2 days at the ideal temperature. After incubation the colony forming units (cfu) are counted and multiplied by the according dilution factor, to enumerate the number of viable cells in the original sample. As this technique is time consuming and quite large standard deviations are obtained, an alternative technique was used in this work.

Growth of bacteria can be monitored via turbidity of a bacterial cell culture and the Bioscreen-C apparatus (Begot et al. 1996) specifically allows to automatically monitor the bacterial growth of several samples using a microwell plate maintained at the required growth temperature. Via this apparatus the growth is followed via turbidity measurements in function

of time. Samples need to be shaken before each turbidity measurement to ascertain a homogeneous mixture. In this research work the Bioscreen turbidity method is used to assess the bacterial survival rate after processing based on the growth rate of the bacteria. At the start of the study the assessment of viability via the Bioscreen turbidity method was validated.

As bacteria were released from the layered pellets using vortex mixing in sterilised water, the influence of vortex time and dissolution volume on the viability was measured. However, viability did not depend on these variables (Table 2). For all experiments during this project the vortex time was set at 30 s in a dissolution volume of 1 ml.

Table 2 Viability ± SD (%) after 10, 30 or 60 s vortexing of layered pellets in 1 or 10 ml sterile water

Vortex time (s)	Dissolution volume (ml)	Viability (%)
10	1	8.2 ± 0.5[a]
30	1	8.1 ± 0.1[a]
60	1	8.0 ± 0.1[a]
30	10	9.1 ± 0.7[a]

[a] Groups with the same superscript are not significantly different ($p>0.05$) (one-way ANOVA, post hoc Scheffé)

The day-to-day variability of a sample analysed via the Bioscreen method on 6 consecutive days using the same batch of growth medium was limited: the overall mean viability was 20.1 % with a coefficient of variation of 5.4 %.

During sterilisation the quality of a growth medium can decrease due to the high temperature causing degradation of sugars and proteins (Wang and Hsiao 1995). It was obvious that the quality of the growth medium had a significant influence on the viability of a sample: growth medium autoclaved for 15 min resulted in a viability of 20.1 ± 1.1 %, whereas medium sterilised for 1 h resulted in a viability of only 10.2 ± 1.3 % for the same sample. The measured $OD_{50\%}$ for a sample not only depended on the number of viable bacteria in the sample, but also on the quality of the growth medium used.

The influence on viability using different batches of sterilised M17 was assessed by analysing a sample on 5 different days using a new batch of sterilised growth medium for each analysis. Similar results were obtained on each day: the overall mean was 21.0 % with a coefficient of variation of 7.1 %.

2.3.2. The layering process

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

The viability of *Lactococcus lactis* Thy 12 after atomisation at different pressures through different nozzles was not significantly different from 100 % at each condition tested. It can be concluded that the shear stress caused by atomisation did not influence the viability of *L. lactis* Thy 12. Fu and Etzel (1995) reported no loss in survival after atomisation of a *L. lactis* C2 strain, and concluded that atomisation alone did not appear to cause cellular injury. 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-based pellets using a 5 % trehalose or lactose matrix: 6.7 ± 1.3 % and 6.3 ± 1.1 % respectively, for trehalose and 8.9 ± 1.7 % and 7.8 ± 1.6 % for lactose (Fig. 3).

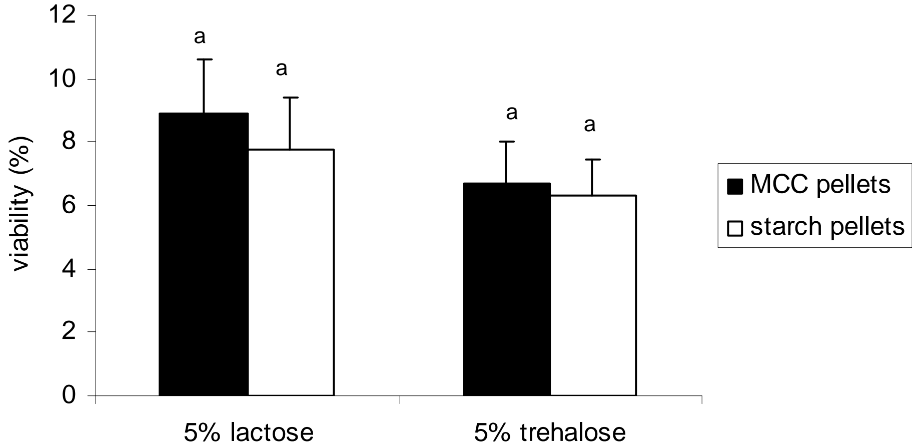


Figure 3 Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in different matrices on different inert carriers (n= 3). (a) Groups with the same superscript within the same stabilising matrix are not significantly different ($p>0.05$) (independent sample T-test)

Since the effect of stabilisers on the viability depended on the bacterial strain and the drying technique (Hubalek 2003), several polyols (disaccharides, polyalcohols, polysaccharides), PVA 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* Thy12 during 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 (Fig. 4). A better stabilisation of bacteria using lactose alone instead of skim milk was also found during freeze-drying by Costa et al. (2000). Probably Ca-caseinate, due to its high 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 (Crowe et al. 1998).

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 in viability (0.3 ± 0.1 %).

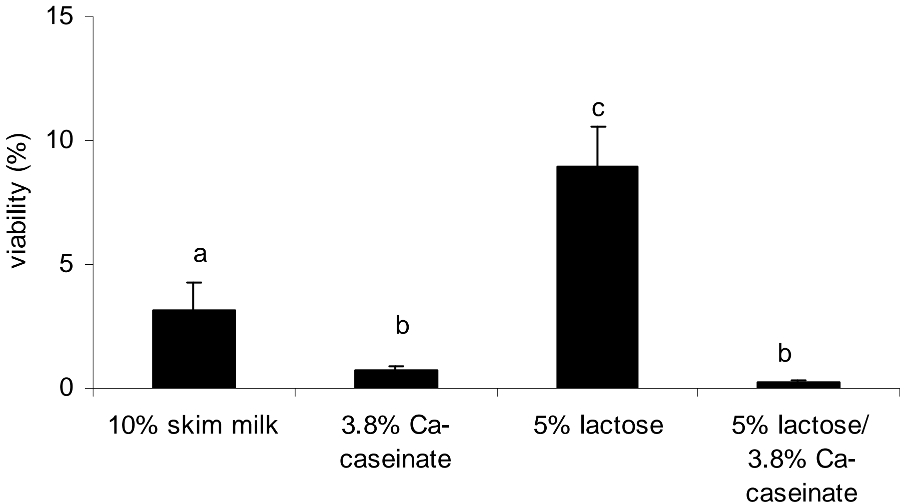


Figure 4 Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in different stabilising matrices (n= 3). (a-c) Groups with the same superscript are not significantly different (p>0.05) (one-way ANOVA, post hoc Scheffé)

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 that in a 5 % lactose matrix (Fig. 5). However, the isomalt and sucrose matrices resulted in sticking problems during layering. Trehalose, known as the golden standard for stabilisation during anhydrobiosis (Crowe et al. 1996), did not result in a better stabilisation after layering. Although Efiuvwevwere et al. (1999) reported a high viability after drying of *L. lactis* using mannitol as stabiliser, this was not confirmed in our study since layering in a 5 % mannitol matrix did not provide protection, probably due to crystallisation of mannitol.

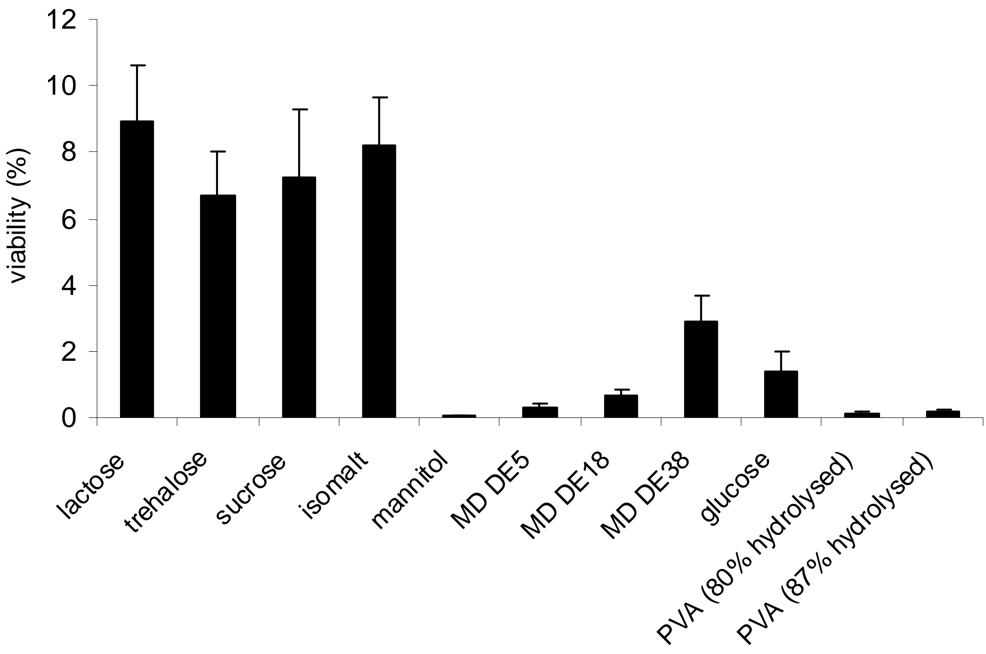
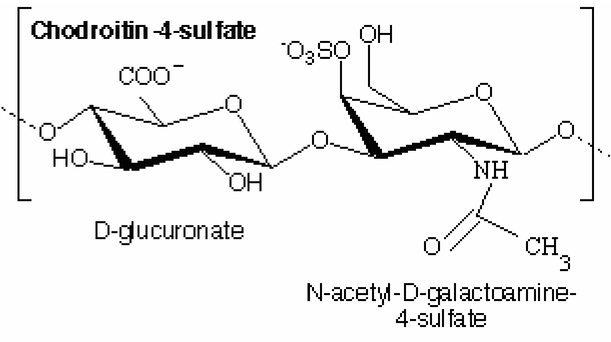


Figure 5 Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in different matrices (5 %) (n=3)

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. 5). However, the viability was much lower compared to that in disaccharide matrices. It was concluded that 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 (Ananta et al. 2005). Completely hydrolysed starch (i.e. 5 % glucose) resulted in a significantly lower viability of 1.38 ± 0.59 %, compared to MD DE 38 (T_g 67-73°C), probably because of its low T_g (21-39 °C). At a temperature below T_g the material is in a glassy state of high viscosity, inhibiting chemical and biochemical reactions as the mobility in this state is very low. At a storage temperature above 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.

Layering of the bacteria in synthetic polyvinyl alcohol matrices resulted in low viabilities (Fig. 5). Although Deaker et al. (2007) showed stabilization of rhizobia using synthetic polyvinyl alcohol matrices (with the highest survival for an intermediate hydrolysed form of PVA), layering using different PVA grades could not protect *L. lactis* during layering.

Peptidoglycan is an essential and specific component of the bacterial cell wall found on the outside of the cytoplasmic membrane of almost all bacteria. The main function of peptidoglycan is to preserve cell integrity. Peptidoglycan consists of glycan strands cross-linked by short peptides. The glycan strands consist of alternating N-acetylglucosamine and N-acetylmuramic acid (Vollmer et al. 2008). Chondroitin sulphate is a sulphated glucosaminoglycan composed of alternating N-acetylgalactosamine and glucuronic acid. Chondroitin sulphate is one of the main compounds of the extracellular matrix and it serves to assure the integrity of the surrounding tissues (Monfort et al. 2008). As there is a similarity between the chemical structure of peptidoglycan and chondroitin sulphate (Fig. 6) it was evaluated if chondroitin sulphate can protect *L. lactis* during drying. However, after layering with 5 % chondroitin sulphate a viability of only 0.15 % was achieved.



a

b

Figure 6 (a) chondroitin sulphate and (b) peptidoglycan structure

Vitamin C as anti-oxidant can be added as an additional stabilizer in combination with sugars to improve the bacterial survival after drying (Champagne et al. 1991). Addition of 1.25 % vitamin C to a 5 % lactose matrix resulted in a low viability (< 1 %) and sticking problems were observed during processing.

During layering in a conventional fluidized bed system, particles reside mainly in the annular zone (Fig. 7). Since particles can become overheated in this zone, which would decrease the bacterial survival, El mafadi et al. (2005) developed a modified fluidized bed system, where hot air only passed through the centre of the bottom plate. When a conical disk was placed over the outer cylinder of the bottom plate, they observed no influence on particle movement and a decrease in temperature in the annular region.

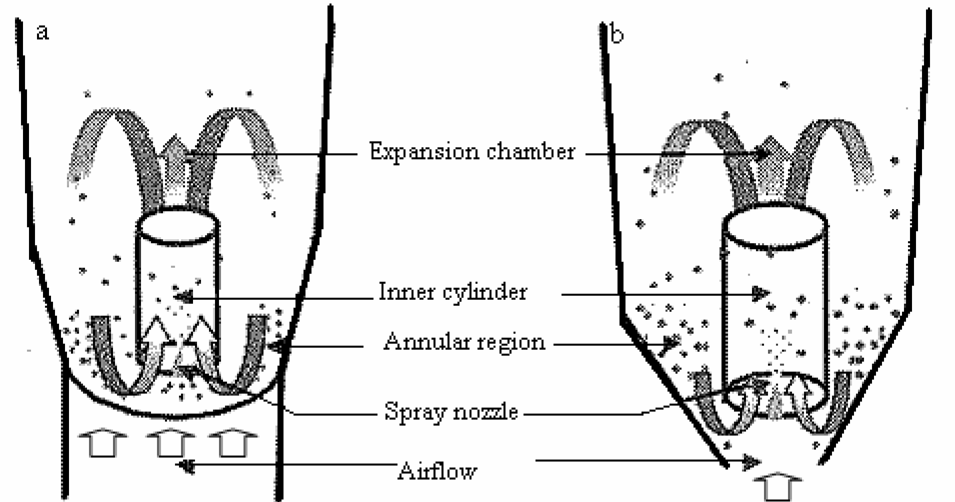


Figure 7 Schematic representation of the Wurster coating process with (a) the conventional equipment (b) modified equipment (El Mafadi et al. 2005)

However, modifying the fluid bed used in this study by placing an insert over the bottom plate (Fig. 8) did not improve the viability using 5 % trehalose as stabilising matrix: after 30 min layering a viability of 6.7 ± 1.3 % was obtained for the conventional system and 4.8 ± 0.3 % for the modified system (Fig. 9).

a b

Figure 8 Bottom device of the Wurster-based fluid bed with (a) the conventional equipment (b) modified equipment

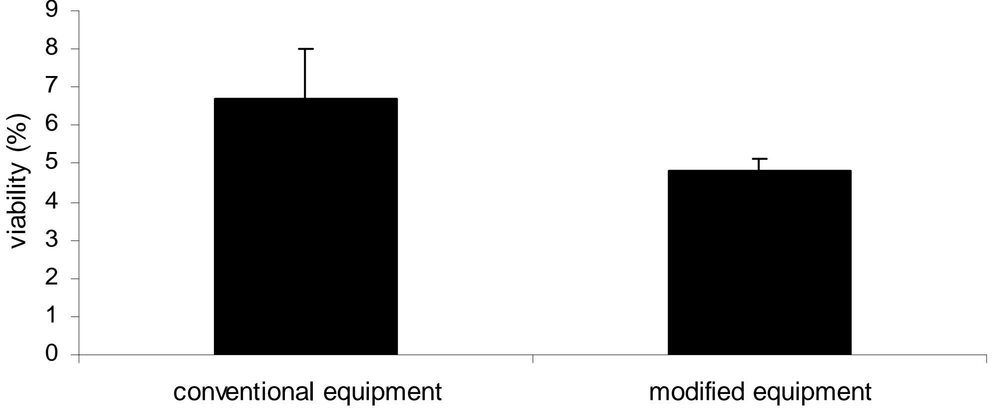


Figure 9 Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in a conventional and modified fluid bed (n= 3) using 5 % trehalose as stabilising matrix.

To evaluate the influence of the stabilizer/bacteria ratio on the viability of *L. lactis* the concentration of skim milk, lactose and trehalose in the layering matrix was varied. Varying the skim milk concentration from 5 to 20 % did not result in a significant difference in viability after layering (Fig. 10). 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 %.

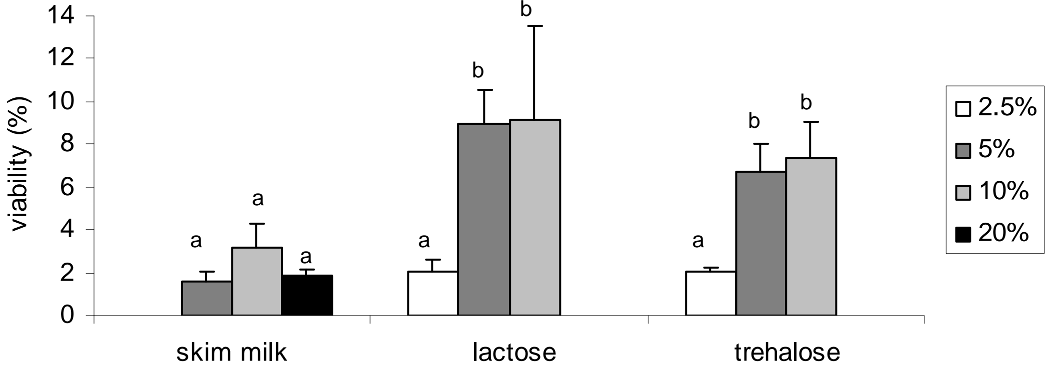


Figure 10 Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in different matrices at different stabiliser/bacteria ratios (n=3). (a,b) Groups with the same superscript within the same stabilising matrix are not significantly different (p>0.05) (one-way ANOVA, post hoc Scheffé)

To assure 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°C or -20°C (Fig. 11). 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).

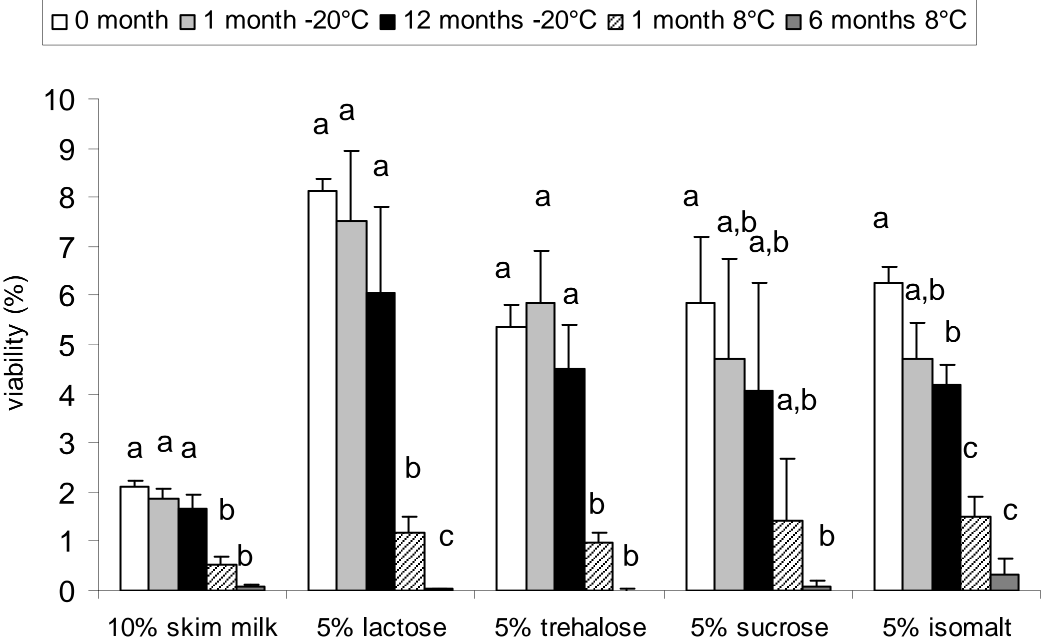


Figure 11 Viability (mean ± SD) of *L. lactis* Thy 12 after layering and after 1, 3, 6 and 12 months storage in Alu sachets (sealed at 20 % RH) at 8 or -20°C. (a-c) Groups with the same superscript within the same stabilising matrix are not significantly different ($p>0.05$) (one-way ANOVA, post hoc Scheffé)

2.3.2.2. 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 for a formulation containing 5 % lactose. Increasing the process time from 30 to 120 min reduced the viability from 9.6 ± 1.7 % after 30 min to 2.9 ± 0.9 % after 120 min. Moreover, sticking of the pellets occurred after 1 h processing. These observations are probably due to crystallisation of lactose during the layering process, damaging the bacterial cell membrane. To inhibit lactose crystallisation, different stabilisers were added to the lactose matrix (maltodextrins and skim milk). To evaluate which components could inhibit lactose crystallisation, 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). Incubation of layered pellets with 5 % lactose as stabilising matrix during 2 h significantly decreased the viability (2.8 ± 0.9 % after 120 min) (Fig. 12). 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. Joupilla and Roos (1994) proposed as hypothesis that the proteins in skim milk protected lactose from crystallisation.

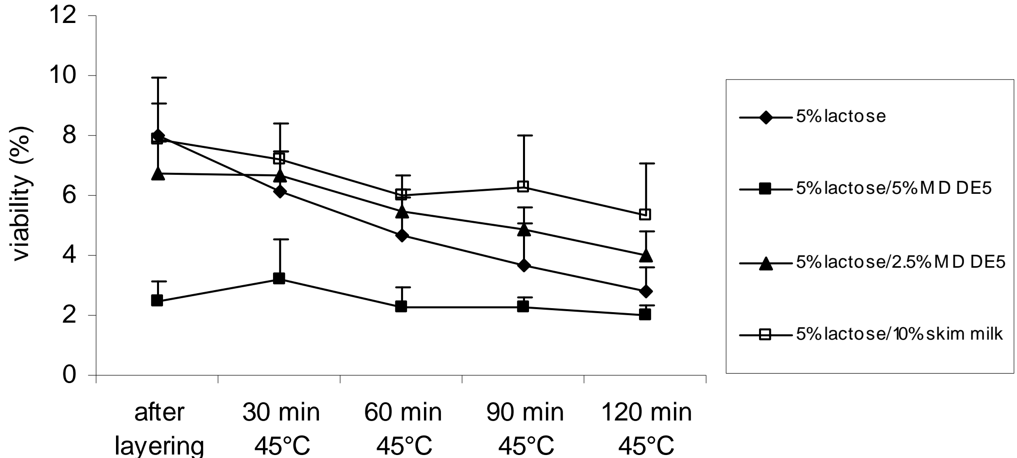


Figure 12 Viability (mean ± SD) of *L. lactis* Thy 12 after layering and subsequent incubation at 45°C for 30, 60, 90 and 120 min in Alu sachets (sealed at 20% RH) (n=3)

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 stabilizing 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. 13). 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 13.6 ± 0.5 % for a 10- and 50-fold concentrated suspension, respectively.

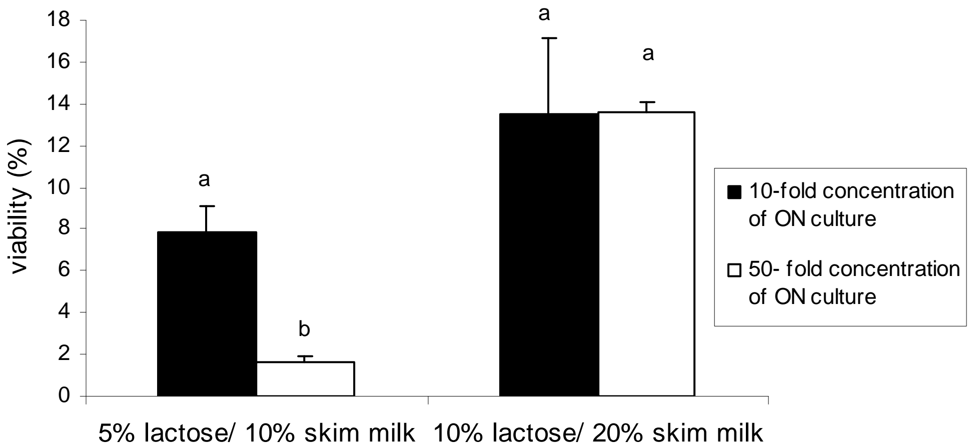


Figure 13 Viability (mean ± SD) of *L. lactis* Thy 12 after 30 min layering in function of bacterial concentration and stabilising matrix/bacteria ratio (n=3). (a,b) Groups with the same superscript within the same stabilising matrix are not significantly different ($p>0.05$) (one-way ANOVA, post hoc Scheffé)

This 10 % lactose/20 % skim milk matrix was also suitable for a 2 h layering process, as a 30-fold 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 relative viability corresponded to 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 of 6 g pellets should be taken. After 12 months storage of these pellets at -20°C no significant decrease in viability was observed.

2.3.2.3. Dynamic vapour sorption

It was hypothised that stabilisation of the bacteria in the lactose/skim milk matrix was partly due to the amorphous state of the sugar layer. As the sugar layer was too thin to use differential scanning calorimetry or X-ray diffraction to determine the crystallinity, DVS was used as alternative technique. When the relative humidity was increased from 55 to 65 % a sudden weight drop was observed due to water loss (Fig. 14). As water sorption below 55 % RH plasticised the amorphous regions of the matrix the glass transition temperature decreased which allowed sufficient molecular movement to initiate crystallisation of the amorphous regions (Buckton and Darcy 1995). During crystallisation the excess of water is expelled from the sample. Since in the isothermal sorption plot of the unlayered MCC pellets, no crystallisation process was observed, the decrease in mass originated from the sugar layer on the pellets. This DVS plot confirmed that the applied lactose/skim milk layer contained amorphous regions which offered protection to the bacteria (Crowe et al. 1998).

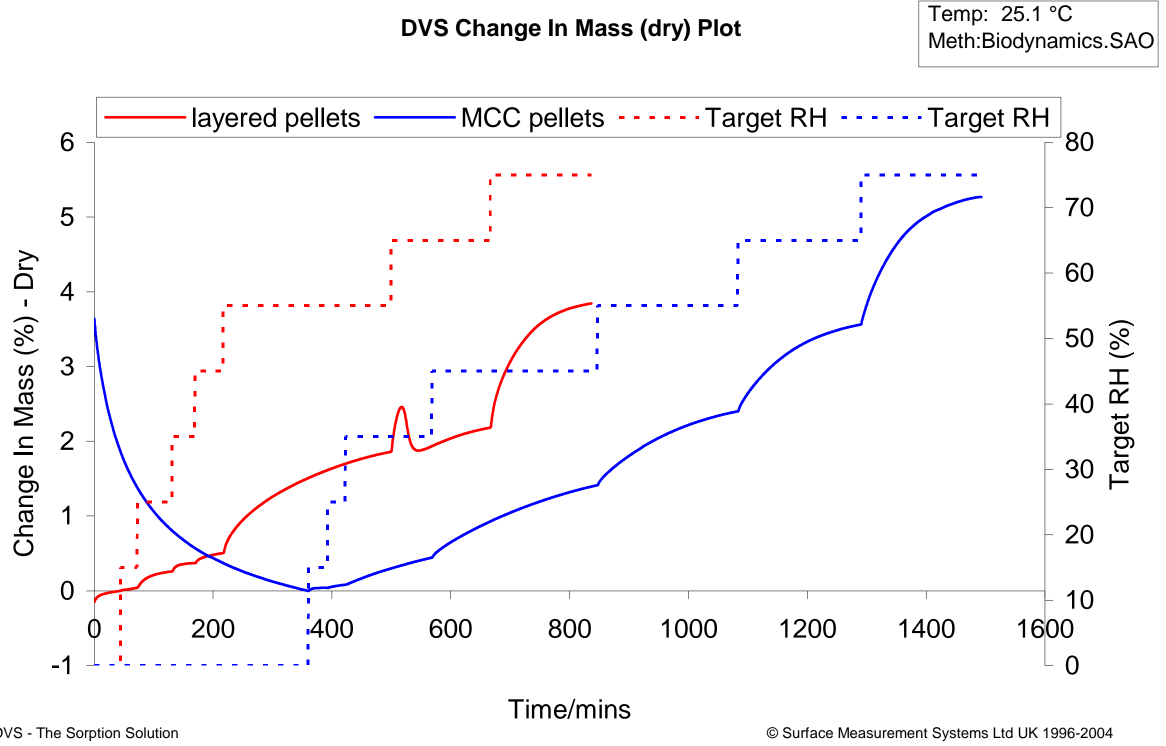


Figure 14 DVS isothermic sorption curve of lactose/skim milk layered and inert pellets

2.3.3. Microencapsulation process

Although immediately after encapsulation there was no loss of bacteria (110.8 ± 14.2 %), only 0.17 % of the bacteria survived the freeze-drying process if only sodium alginate was used as encapsulation polymer. To increase the viability after drying 5 % lactose or trehalose were added to the sodium alginate solution to stabilize the encapsulated bacteria during drying. Immediately after encapsulation no loss in viability was found under these conditions. After drying 11.7 ± 4.7 % of the bacteria remained viable in the lactose-stabilised microcapsules and 8.1 ± 3.1 % in the trehalose-stabilised capsules. Via the plating out method an absolute number of viable bacteria of respectively 6.6×10^8 cfu and 1.2×10^9 cfu per 100 mg microcapsules was measured. To verify if the freezing rate had a detrimental effect on the bacterial viability, microcapsules were flash frozen using liquid nitrogen. Immediately after freezing of the lactose- and trehalose-stabilised capsules, 53.5 ± 6.0 % and 49.9 ± 10.8 %, respectively, of the bacteria were viable. After drying the viability was about 10 % for both type of microcapsules, indicating that the survival rate could not be improved via immediate freezing. In order to assure that, during microcapsule hardening, there was no loss of stabiliser due to diffusion into the $CaCl_2$ solution, lactose and trehalose were also added in a 5 % concentration in the $CaCl_2$ solution. This resulted in an absolute bacterial load of 9.7×10^8 cfu and 1.1×10^9 cfu/100 mg, which is similar to the experiment without stabiliser in the hardening solution. There was no difference between lactose and trehalose as stabilising molecules. The number of viable bacteria in the microcapsules was equal to the layered pellets. However, the obtained microcapsules were very brittle and sticky to handle (Fig. 15). A longer freeze-drying process did not result in a better microcapsule quality.

Figure 15 Microcapsules before drying (left) and after drying (right)

2.4. 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 6 g pellets corresponded with 10^{11} cfu. 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 stabilise 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. Microencapsulation did not result in an acceptable multi-particulate formulation for the bacterial strain under evaluation.

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3

ENTERIC PROTECTION AND ILEUM TARGETING OF A LAYERED PELLET FORMULATION

3.1. Introduction

In the previous chapter layering of pellets was presented as a valuable technique for the production of a viable *L. lactis* dosage form. This multi-particulate dosage form offered several advantages: (a) layering and enteric coating (required due to the sensitivity of *L. lactis* to gastric fluids (Klijn et al. 1995)) can be performed in the same equipment; (b) the small particle size of the pellets assures fast gastric emptying of the formulation, limiting the contact time with the gastric environment (Krämer and Blume 1994); (c) ease of administration since small particles can be easily swallowed or mixed with food. In this chapter 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 ileal part of the intestine, the ileum targeting capacity of the coating polymer was assessed using

different coating thicknesses. In another approach the enteric polymer Eudragit® FS30D was chemically modified to achieve ileum targeting.

3.2. Materials and methods

3.2.1. Materials

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). Sureteric® (Colorcon, Kent, UK) was also used for enteric coating. HPMC-based (Opadry®) (Colorcon, Kent, UK) or PVA-based polymers (Opadry® II and Opadry® AMB) (Colorcon, Kent, UK) were used as subcoatings before enteric coating. Microcrystalline cellulose spheres (Cellets®, Pharmatrans-Sanaq, Basel, Switserland) were used as inert carriers for the layering and coating experiments. Thymidine (Alkemi, Lokeren, Belgium) and microcrystalline cellulose (Avicel® PH 101, FMC Europe, Brussels, Belgium) were used for the production of the marker pellets.

3.2.2. Enteric coating and subcoating of the layered pellets

Layered pellets were enterically 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).

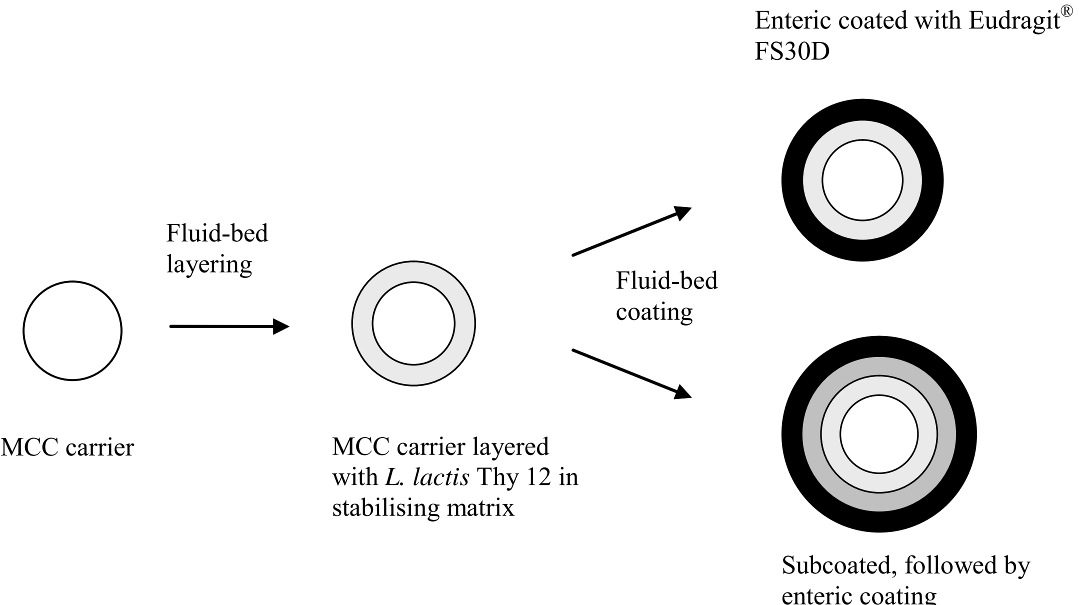

Figure 1 Flow-chart for layering, subcoating and coating of the pellets

For 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 (Silverson, Bucks, United Kingdom) until a fine, homogenous dispersion was obtained. This dispersion was gently added to the Eudragit® FS30D dispersion and mixed using magnetic stirring. The coating dispersion was passed through a 0.3 mm sieve before use. Throughout the coating process the coating dispersions were stirred. 300 g pellets (Cellets®) were coated in a fluid bed coating apparatus (GPCG1, Glatt, Binzen, Germany), used in the bottom spray mode with Wurster setup (nozzle Ø 0.8 mm). Before coating, the pellets were preheated to the required product temperature. Pellets were enterically 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 at -20°C.

Sureteric® coating dispersion (mixture of polyvinyl acetate phthalate, plasticisers and other processing ingredients) was prepared by gently adding the powder to water on a magnetic stirrer until a 15 % dispersion was obtained. To prevent foam formation a simethicone emulsion was added in a concentration of 0.33 % of the polymer dry weight.

To prepare the subcoating dispersions, the powders were gently mixed with 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 as matrix) were subcoated with 4 % Opadry®, Opadry® II and Opadry® AMB prior to enteric coating. The coating and subcoating parameters are shown in Table 1.

Table 1 Process parameters used for enteric-coating 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

3.2.2.1. 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 0.0125M phosphate buffer was used because a more concentrated phosphate buffer had a detrimental effect on the viability of the bacteria. To evaluate the viability after gastric passage of the enterically coated pellets, a dissolution test during 2 h in 0.1 N HCl was performed using the reciprocating cylinder method (Bio-dis, Vankel, NJ, USA) (USP apparatus 3) with 1 g pellets per 250 ml vessel. Afterwards the pellets were stirred 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 plate count method. Via the pour plate 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.

3.2.3. Ileum targeting

3.2.3.1. Influence of coating thickness on release profile

3.2.3.1.1. Production of thymidine pellets

To evaluate the impact of the coating thickness of Eudragit® FS30D on the release profile from the pellets, thymidine was incorporated in pellets (1 %) prepared by extrusion/spheronisation. Thymidine was selected as marker because of its easier quantification than that 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) 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.

3.2.3.1.2. 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, New Jersey, US) sealed at 20 % RH and stored at -20°C.

3.2.3.1.3. Thymidine release from 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). Pellets were consecutively exposed to two media: 0.1 N HCl (2 h) and a 0.05 M phosphate buffer at pH 6.8, 7.0 or 7.4 (3 h 40 min). The concentration of thymidine in both dissolution media was measured spectrophotometrically (UV-1650PC, Shimadzu, Antwerp, Belgium) at 267 nm.

3.2.3.2. Chemical modification of Eudragit® FS30D

3.2.3.2.1. Preparation of chemically modified Eudragit® FS30D polymer

The commercially available Eudragit® FS30D dispersion was added to a water/methanol mixture (ratio: 50/50, v/v) in a 25/75 (v/v) ratio of Eudragit® FS30D versus the solvent mixture. Different amounts of NaOH (Table 2) were added and the solution was gently mixed during 18 h using a magnetic stirrer. After 18 h the polymer was precipitated by adding 0.2 N HCl and washed with distilled water. The precipitated polymer was frozen using dry ice in aceton, followed by freeze-drying (Lyolab 3000, Heto Lab Equipment, Allerod, Denmark). Afterwards the dried polymer was crushed and the powder was sieved to collect the fraction < 180 μm.

Table 2 Different types of chemically modified Eudragit® FS30D

	Eudragit® FS30D	**Low level NaOH (LL_{NaOH})**	**Medium level NaOH (ML_{NaOH})**	**High level NaOH (HL_{NaOH})**
Amount NaOH (mmol/L)	0	94	188	376

3.2.3.2.2. Assessment of the number of methacrylic acid units

The number of methacrylic acid units was assessed via a colorimetric titration (European Pharmacopoeia 6). 5 g polymer (using Eudragit® FS30D as well as the chemically modified polymers) was dissolved in isopropyl alcohol/water (ratio: 90/10, v/v). NaOH 0.5 N was used as titrant and phenolphthalein as colour indicator. 1 ml of 0.5 N NaOH corresponds with 43.045 mg methacrylic acid units.

$$\text{Methacrylic acid units on dry substance (\%)} = \frac{\text{x ml 0.5N NaOH} \times 43.045}{\text{Sample weight (mg)}} \times 100$$

According to the manufacturer's specifications, the number of methacrylic acid units of a commercially available Eudragit® FS30D dispersion is between 9.2 and 12.3 % (calculated on dry substance).

3.2.3.2.3. Evaluation of the ileum-targeting potential of the polymers

To assess the ileum targeting potential of the polymers (using Eudragit® FS 30D as well as the chemically modified polymers) a 10 % polymer solution was prepared in an ethanol/acetone mixture (50/50, v/v) and hard gelatin capsules were dip-coated using these polymer solutions. The hard gelatin capsules were filled with a lactose/methylene blue mixture, using methylene blue as marker for capsule disintegration. Different polymer layers were applied onto the capsules by repeatedly dipping them in the polymer solution and drying between each dipping step. First, 3 layers were applied on the body of the capsule, followed by 4 layers on the cap

and one additional layer on the body. Disintegration of the capsules (indicating dissolution of the polymer film) was assessed via an *in vitro* disintegration test using the apparatus as described in the European Pharmacopoeia (PTZ-E Pharma Test, Hainburg, Germany). After an initial 2 h in 0.1 N HCl as test medium (37°C, using disks) for disintegration, the capsules were transferred to a 0.05 M phosphate buffer (having a pH of 5.5, 6.8, 7.0 or 7.4) to assess the ileum-targeting potential based on the capsule disintegration in these buffers.

3.3. Results and discussion

3.3.1. 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) (see chapter 2 for details) and enterically coated with Eudragit® FS30D. Since enteric coating reduced viability (Table 3) with increasing coating thickness, there is a detrimental effect of the coating process due to an interaction between *L. lactis* Thy 12 and the enteric coating. This drop in viability could not be linked to a longer exposure time to higher temperatures during processing as pellets fluidized for the same time at the coating temperature showed no decrease of viability. Moreover, after 2 h in 0.1 N HCl (simulating the gastric residence time) viability further decreased; only about 1 % of the bacteria remained viable in the coated formulation independent of coating thickness. To avoid direct contact between the bacteria and the enteric coat, subcoatings were applied onto the pellets before enteric coating. When formulating drugs like omeprazole, subcoatings have been used successfully to prevent direct contact between acid-labile drug and the acidic functional groups of the enteric coating (Stroyer et al. 2006). In 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).

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

<table>
<tr><th></th><th>After layering</th><th>Subcoating</th><th>Enteric coating</th><th>After 2 h 0.1N HCl</th></tr>
<tr><td>No subcoating</td><td rowspan="4">12.4 ± 1.4</td><td>/</td><td>5%: 8.6 ± 2.8
10%: 4.5 ± 1.8
15%: 2.1 ± 0.5</td><td>1.1 ± 0.4
0.6 ± 0.2
0.4 ± 0.4</td></tr>
<tr><td>Opadry®</td><td>3.7 ± 0.5</td><td>/</td><td>/</td></tr>
<tr><td>Opadry® AMB</td><td>3.2 ± 1.8</td><td>/</td><td>/</td></tr>
<tr><td>Opadry® II</td><td>12.5 ± 2.9</td><td>5 %: 2.9 ± 1.1</td><td>0.9 ± 0.3</td></tr>
</table>

A coating thickness of 5 % was selected as a thicker Eudragit® FS30D coat resulted in a decrease of viability (Table 3). The viability of pellets after 2 h layering with a concentrated bacteria suspension and after coating of these layered pellets with 5 % Eudragit® FS30D was 13.7 and 7.4 %, respectively. This corresponds to an absolute number (using the plate count method) of viable bacteria of $1.7 \times 10^9 \pm 1.2 \times 10^8$ cfu and $9.9 \times 10^8 \pm 8.8 \times 10^7$ cfu/100 mg pellets, for the layered and coated pellets, respectively. Hence about 10 g pellets is required to administer a dose of 10^{11} cfu. After 2 h in 0.1 N HCl the viability decreased approximately 1 log unit ($1.4 \times 10^8 \pm 6.0 \times 10^7$ cfu/100 mg).

As acrylic-based enteric polymers were toxic for the bacterial survival, another enteric polymer was evaluated. Sureteric®, a polyvinylacetate-phthalate polymer, was selected despite the fact that it already dissolves at pH 5.5 which is too low for ileum targeting. After coating viability decreased from 10.5 ± 3.7 % to 3.3 ± 1.7 % and 2.6 ± 1.3 % for a weight

gain of 5 and 10 %, respectively. Sureteric® yielded an even lower viability after coating, compared to the Eudragit® FS30D polymer (Fig. 2).

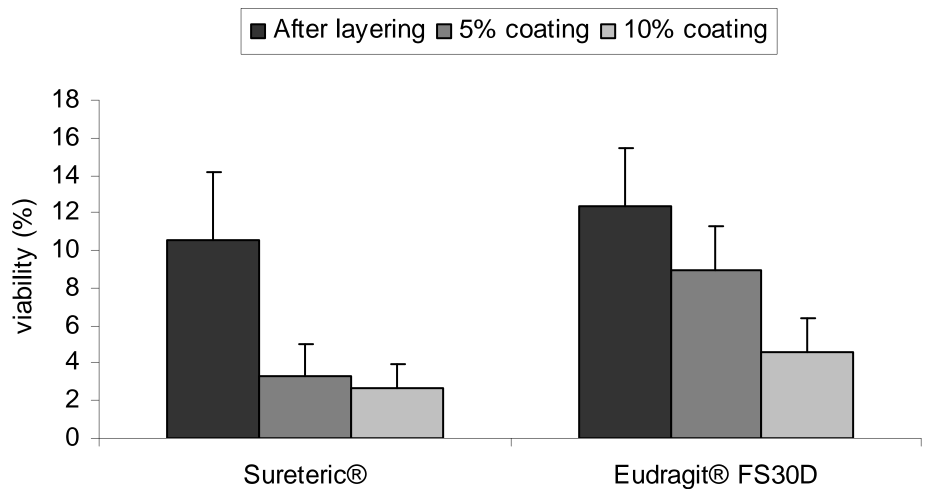

Figure 2 Viability ± SD (%) after layering and coating with Sureteric® and Eudragit® FS30D

Probably the acidic functional groups, present in both polymers (Fig. 3), are responsible for the decrease in viability.

a

b

Figure 3 Chemical structure of (a) Eudragit® FS30D and (b) Sureteric®

3.3.2. Ileum targeting

The release of the IL-10 producing bacteria must be targeted to the ileum, as the ileum is the major site of inflammation in patients with Crohn's disease. Previous results (Huyghebaert et al. 2005) showed that the enteric polymers Eudragit® L30D55 and Eudragit® FS30D did not result in ileum targeting (i.e. release from pH 6.8): a Eudragit® L30D55 coating (thickness corresponding to 30% polymer weight gain) dissolved at a pH lower than 6.8, while a Eudragit® FS30D (thickness corresponding to 15 % polymer weight gain) only dissolved at a pH above 7.4. An *in vivo* study showed that HPMC-capsules coated with 15 % Eudragit® FS30D resulted in 30 % of the cases in a release distaly from the ileum target site (Vanhoutvin et al. 2007).

A formulation coated with 5 % Eudragit® FS30D, selected based on its low toxicity towards *L. lactis* (Table 3), was evaluated for its ileum targeting capacity, compared to pellets coated with 15 % polymer. At pH 6.8 thymidine release (used as marker) was slow with a slightly faster release for the 5 % coated pellets (Fig. 4). In phosphate buffer pH 7.0 complete thymidine release was obtained after 100 min from pellets coated with 5 % Eudragit®, whereas pellets with a 15 % weight gain only released 50 % of the marker after 210 min. The influence of coating thickness on release rate was less pronounced at pH 7.4. These results indicated that ileum targeting could be improved by reduction of the coating thickness from 15 to 5 %.

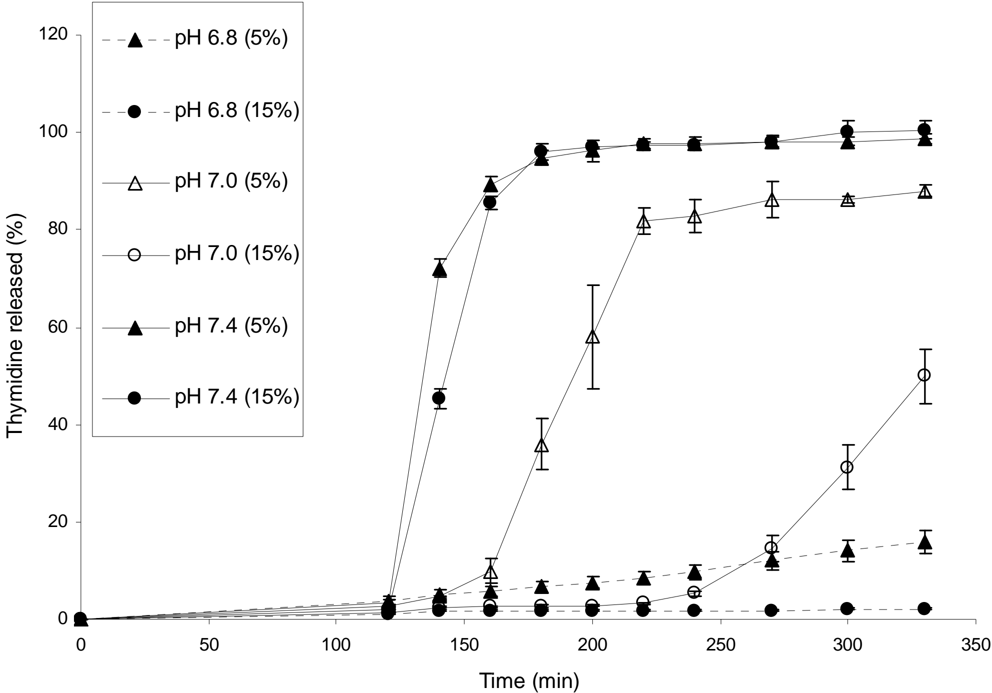

Figure 4 Release profiles of thymidine (mean ± S.D., n=3) from pellets coated with 5 (▲) and 15 % (●) Eudragit® FS30D after 2 h immersion in HCl 0.1 N and subsequently in buffer solution with pH 6.8 (---, black symbols), 7.0 (—, open symbols) and 7.4 (—, black symbols)

In another approach to obtain ileum targeting the commercially available Eudragit® FS30D polymer was chemically modified to increase the number of free carboxylic groups. Since the dissolution characteristics of anionic copolymers based on methyl acrylate, methyl methacrylate and methacrylic acid are determined by the number of free carboxylic groups, the objective of the chemical modification of Eudragit® FS30D was to induce release at a lower pH. For the Eudragit® FS30D polymer the ratio of free carboxyl groups to ester groups is approximately 1:10, for Eudragit® L30D-55 the ratio is 1:1, corresponding to a release starting at pH 7.4 and 5.5, respectively. Since release at pH 6.8 is required for ileum targeting, neither type of Eudragit® is optimal. Hence it was the objective to increase the number of methacrylic acid units of Eudragit® FS30D (via hydrolysis of the ester groups using a strong base) to improve its dissolution at lower pH (Fig. 5). Addition of a strong acid after the

saponification reaction decreased the aqueous solubility of the polymer and the chemically modified polymer can be collected and washed.

$$R\text{-}C(=O)\text{-}O\text{-}R' + NaOH \xrightarrow[\text{or hydrolysis}]{\text{saponification}} R\text{-}C(=O)\text{-}O^{-}Na^{+} + HO\text{-}R'$$

carboxylate ester + sodium hydroxide → sodium carboxylate + alcohol

Figure 5 Hydrolysis or saponification of a carboxylate ester

Different amounts of NaOH were added to Eudragit® FS30D resulting in 3 grades of modified polymer, characterised by a different number of free carboxylic groups (Fig. 6). The unmodified polymer had 10.0 % methacrylic acid units (on polymer dry weight) (corresponding with the specification of the manufacturer) and the free carboxylic groups increased in function of the NaOH concentration during chemical modification: 12.2, 21.8 and 39.0 % using 94, 188, 276 mM NaOH, respectively. Based on the fraction of free COOH-groups these chemically modified polymers are potential candidates for ileal targeting since the percentage of methacrylic acid groups is between the values for Eudragit® L30D55 (46.0-50.6 %, having a too fast release) and Eudragit® FS30D (9.2-12.3 %, having a too slow release).

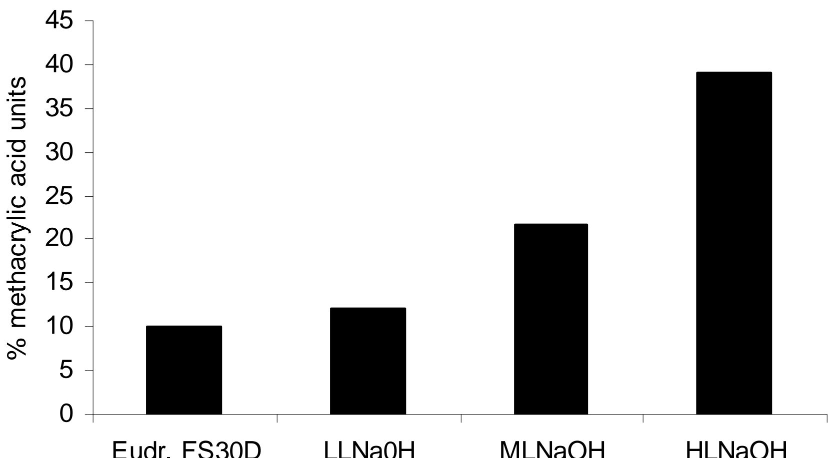

Figure 6 Percentage of methacrylic acid units (on polymer dry weight) in unmodified and modified Eudragit® FS30D polymers. Eudragit® FS30D was chemically modified using different NaOH concentrations for saponification of the ester groups: 94, 188 and 276 mM for LL_{NaOH}, ML_{NaOH} and HL_{NaOH}, respectively.

Capsules filled with methylene blue as marker were dip-coated with these polymers dissolved in organic solvents and its enteric properties (ability to withstand 2 h in 0.1N HCl) as well as the pH required for disintegration were assessed. To obtain ileum targeting the capsule should remain intact below pH 6.8 and disintegrate at pH 6.8 within 60 min. The disintegration properties of the coated capsules are summarized in Table 4. All coated capsules had sufficient enteric properties as no disintegration occurred within 2 h in 0.1 N HCl. As expected the capsules coated with unmodified polymer (Eudragit® FS30D) only disintegrated at pH 7.4. When the polymer chemically modified with the lowest concentration of NaOH (LL_{NaOH}) was used no disintegration occurred at pH 6.8, however the capsules opened after 20 min at pH 7.0. Using an intermediate NaOH concentration (ML_{NaOH}) the capsule opened after 20 min in pH 5.5. As the ML_{NaOH} polymer already released its content at a pH too low for ileum targeting, the polymer chemically modified with the highest NaOH concentration (HL_{NaOH}, containing the highest fraction of free carboxylic groups) was not tested.

Table 4 Disintegration times ± SD (min) of capsules (n=3) coated with Eudragit® FS30D and polymers modified using different concentrations of NaOH (LLN_{aOH} and ML_{NaOH})

	Eudragit® FS30D	**LL_{NaOH}**	**ML_{NaOH}**
0.1N HCl	No disintegration (2h)	No disintegration (2h)	No disintegration (2h)
pH 5.5	No disintegration (1h)	No disintegration (1h)	20 ± 4 min
pH 6.8	No disintegration (1h)	No disintegration (1h)	-
pH 7.0	No disintegration (1h)	20 ± 2 min	-
pH 7.4	55 ± 5 min	-	-

- : not tested

3.4. Conclusion

Layered pellets were protected by Eudragit® FS30D against the gastric fluid, resulting in an acceptable cell load of 1.4×10^8 cfu/100 mg after gastric passage. The enteric polymer itself and small amounts of acid that penetrated in the layer of bacteria decreased the bacterial survival. Ileum targeting could be achieved with a thin coat of Eudragit® FS30D, resulting in release at pH 7.0, close to the ileum pH of 6.8. Modification of Eudragit® FS30D by increasing the number of free carboxylic groups, resulted in a polymer suitable for ileum targeting.

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4

IN VIVO EVALUATION OF THE VAGINAL DISTRIBUTION AND RETENTION OF A MULTI-PARTICULATE PELLET FORMULATION

4.1. Introduction

To deliver probiotic bacteria to the vaginal region, layered pellets were proposed as delivery system. However, pellets have not yet been used as vaginal delivery system, hence their behaviour after vaginal administration should be evaluated. The application of pellets for vaginal delivery is not limited to probiotics, but can be extended to microbicides against HIV infection (Garg et al. 2003), to antifungal and antimicrobial drugs for local drug action or to large molecular weight drugs like peptides and proteins (Richardson and Illum 1992) for systemic drug delivery. An optimal vaginal formulation should have (1) a long retention time to maximise drug release, and (2) a proper spreading over the vaginal epithelium to obtain fast absorption or to maximize the effect in case of local treatment, should (3) be easy to administer and (4) not cause discomfort to improve patient compliance. Most of the vaginal formulations are cleared too fast from the vaginal cavity as a result of the self cleansing action

of the vagina and/or because the vaginal formulation failed to attain a proper distribution over the vaginal mucosa (Woolfson et al. 2000). Pellets are herewith proposed as a novel vaginal drug delivery system as it is anticipated that due to their small particle size they will evenly distribute over the vaginal epithelium and will be less sensitive to gravity, resulting in a longer retention time. The *in vivo* behaviour (vaginal distribution and retention) and patient acceptability (irritation, discomfort) of non-disintegrating microcrystalline cellulose (MCC) and disintegrating starch-based pellets were evaluated following administration of these pellets packed in hydroxypropylmethylcellulose (HPMC) or hard gelatin capsules. The vaginal distribution and retention of a freeze-dried powder (lactose/skim milk) packed in a capsule was also evaluated. In addition the influence of the carrier material on the vaginal pH and microflora was verified.

4.2. Materials and methods

4.2.1. Materials

Microcrystalline cellulose (MCC) (Cellets® 500 µm, Pharmatrans-Sanaq, Basel, Switzerland) were used as non-disintegrating pellets. Disintegrating starch-based pellets were prepared via extrusion/spheronisation using a high amylose, crystalline and resistant starch (Uni-Pure®EX starch, National Starch and Chemical Company, New Jersey, USA) as main excipient (concentration 84.9 %). Hydroxypropylmethylcellulose (HPMC) (Methocel® E15 LV EP Pharm, Colorcon, Dartford, UK) (4.9 %) was used as a binder in the pellets and sorbitol (Sorbidex® P 16616, Cerestar, Vilvoorde, Belgium) (10.2 %) was added to modify the consistency of the wet mass (Dukic et al. 2007). Demineralised water was used as granulation liquid. Riboflavin sodium phosphate (Certa, Braine l'Alleud, Belgium) (concentration: 5 %)

was added as a marker to visualise the spreading and to quantify the retention time of the formulation in the vaginal cavity.

4.2.2. Preparation of the pellets

Starch-based pellets were produced by extrusion/spheronisation. Dry mixing was performed in a Turbula® mixer (model T2A, W.A. Bachofen, Basel, Switzerland) for 15 min. The powder mixture was granulated with demineralised water for 10 min using a planetary mixer (Kenwood Chief, Hampshire, UK) with a K-shaped mixing arm. Water was added during the first 30 s of the wet massing phase. To ensure uniform water distribution, the material adhering to the mixing bowl was regularly removed. The wet mass was extruded at an extrusion speed of 50 rpm using a single screw extruder (Dome extruder lab model DG-L1, Fuji Paudal, Tokyo, Japan) equipped with a dome-shaped extrusion screen with 0.4 mm perforations. The extrudates were spheronised in a spheroniser having a friction plate with cross-hatched geometry (Caleva Model 15, Caleva, Sturminster Newton, Dorset, UK). Spheronisation time was 3 min and spheronisation speed 1000 rpm. The pellets were overnight dried in an oven (Memmert, Schwabach, Germany) at 40°C. The size fraction of 315 - 800 µm was separated using a sieve shaker (Retsch, Haan, Germany). Pellets were packed into HPMC (size 00, Vcaps, Capsugel, Bornem, Belgium) or gelatin (size 00, Intercaps, Aca pharma, Waregem, Belgium) capsules. The number of pellets per capsule was about 7500.

4.2.3. Preparation of the freeze-dried powder

An aqueous dispersion of 10 % lactose (α-Pharma, Braine-l'Alleud, Belgium) and 20 % skim milk (Difco, Becton Dickinson, MA, USA) was freeze-dried using an Amsco Finn Aqua GT4 freeze-dryer (Amsco, Germany). The dispersion was frozen to -45°C within 175 min at 1000 mbar. Primary drying was performed at -15°C and at a pressure varying between 0.8 and 1 mbar during 13 h, followed by secondary drying at elevated temperature (10°C) and reduced pressure (0.1–0.2 mbar) for 7 h. The powder was packed into HPMC or hard gelatin capsules (size 00).

4.2.4. In vitro disintegration of pellets and capsules

Simulated vaginal fluid (Owen and Katz 1999) was used as test medium to evaluate the disintegration properties of the pellets using the watch glass method to simulate vaginal disintegration (Yamaguchi et al. 1990a; Yamaguchi et al. 1990b). One g of pellets was placed on the centre of a watch glass (diameter of 11 cm), which floated on a water bath at 37°C. 4 ml simulated vaginal fluid (37°C) was poured on the pellets and disintegration was evaluated by touching the pellets with dumb and index finger at regular time points. The disintegration time was defined as the time point at which the pellets consisted of a soft mass with no palpably firm, unmoistened core. The disintegration time of HPMC and hard gelatin capsules was assessed using the same procedure, the disintegration time was defined as the time point at which the pellets were released from the capsules.

4.2.5. *In vivo tests*

4.2.5.1. Evaluation of vaginal distribution and retention of pellet and powder formulations

Clinical trials were performed with healthy volunteers (5 volunteers in each group). Volunteering women were screened to exclude gynaecological and systemic pathology, including cervico-vaginal infections or vaginal microflora alterations according to a standardized protocol. Only nullipara healthy premenopausal volunteers aged between 18 and 50 year were included in the study. Study participants adhered to a strict protocol that involved an extensive list of behaviours they were to refrain from to avoid interference with the vaginal formulation, including abstinence from coitus for 48 hours prior to and 24 h after administration of the product, no use of vaginal hygiene products (spray, foams,…) and no depilation of pubic hair. To avoid interference with menstrual or withdrawal bleeding, study participants adhered to a continuous oral contraceptive regimen during the conduct of the studies. All *in vivo* experiments were approved by the ethical committee of Ghent University Hospital.

Two pellet and one powder formulation were administered: non-disintegrating MCC pellets, fast-disintegrating starch-based pellets and a freeze-dried lactose/skim milk powder (containing 5 % riboflavin sodium phosphate as marker). These products were administered using HPMC or hard gelatin capsules. Capsules were administered high in the vagina at the fornix posterior using a commercially available applicator (Infemin applicator, Pierre Fabre santé Benelux, Brussels, Belgium). All volunteers remained supine for 3 or 6 h after administration of the formulation and underwent colposcopy after 3, 6 or 24 h. The *in vivo* behaviour of capsules and pellets (disintegration and spreading) was assessed via colposcopy

using photographs (n=12) to monitor the entire ectocervical and vaginal mucosa. Table 1 details the formulations administered to each group of volunteers (n=5) and the time points at which colposcopy was performed.

Table 1 Overview of the study groups used to evaluate the *in vivo* behaviour (disintegration, spreading, retention time) of pellets and capsules after vaginal administration

Group	Formulation	Capsule type	Colposcopy	RSP* marker
1	Microcrystalline cellulose pellets	HPMC	3 h and 24 h	-
2	Starch-based pellets	HPMC	6 h and 24 h	+
3	Starch-based pellets	HPMC	24 h	+
4	Freeze-dried powder	HPMC	6 h and 24 h	+
5	Starch-based pellets	Gelatin	6 h and 24 h	+

* RSP: riboflavin sodium phosphate

Using pellets or powder with riboflavin sodium phosphate (RSP) as marker, the vaginal discharge of pellets and powders was monitored over a period of 24 h using panty shields. After 6, 12 and 24 h the panty shields were replaced and the RSP amount on each panty shield was determined. To monitor the vaginal distribution of the formulation, the fornix, the mid portion of the vault mucosa and the introitus were swabbed during colposcopy and the fraction of RSP detected at the different sites was assessed.

4.2.5.1.1. Analysis of the panty shields and swabs

To determine the amount of riboflavin sodium phosphate, the panty shields and swabs were stirred for 24 h protected from light in 500 and 5 ml demineralised water, respectively. The RSP concentration in a filtered (Spartan 30/0.2 RC membrane filter, Whatman, Schleicher & Schuell, Dassel, Germany) sample was measured spectrophotometrically at 266 nm (UV-

1650PC, Shimadzu, Antwerp, Belgium). The amount of RSP recovered was expressed as the percentage of the total administered RSP dose.

4.2.5.2. Evaluation of the vaginal pH and microflora after administration of starch-based pellets and lactose/skim milk powder

To evaluate the effect of the excipients used as carrier material in the vaginal formulations a double-blind, randomised, parallel study was performed with 24 healthy volunteers: 2 groups of each 8 women followed a 5-day treatment with hard gelatin capsules filled with (a) starch-based pellets and (b) freeze-dried lactose/skim milk powder, whereas no formulation was vaginally administered in the control group (n=8) (i.e. no treatment). The same inclusion and exclusion criteria were used as during evaluation of the vaginal retention time and spreading. In addition no local or systemic antibiotics (including anti-mycotics and anti-protozoics) were allowed during a period of 3 weeks before the start of the study and only volunteers with normal microflora were included. The latter was assessed via vaginal swabbing and analysis of the vaginal microflora.

During the 5-day treatment period one capsule per day was self-administered by the volunteers just before bedtime using a vaginal applicator. On day 14 the intake of the monophasic pill was stopped to cause menstruation.

On day 6 (1 day after administration), 14, 22 (after menstruation) and 35 the volunteer underwent a gynaecological examination. Vaginal pH was measured using a pH test strip (Macherey-Nagel GmbH, Düren, Germany) and the vagina was swabbed with an ESwab (Copan diagnostics, Corona, CA, USA). Swabs were used for Gram-staining and culturing to identify H_2O_2-producing strains at the Laboratory for Bacteriological Research (Ghent University Hospital).

4.2.5.2.1. Gram-staining

Swabs were smeared on a plane glass slide and air-dried at room temperature. The slides were Gram-stained, examined under oil immersion at a magnification of 1000 (Mirastainer, Merck-Belgolab, Overijse, Belgium) and assigned a score according to Verhelst et al. (2005). Accordingly, Gram-stained vaginal smears were categorized:

- grade Ia: only *Lactobacillus crispatus* cell types
- grade Ib: only other *Lactobacillus* cell types
- grade Iab: both *L. crispatus* and other lactobacilli
- grade I-like: Gram positive rods
- grade II (intermediate): *Lactobacillus* and *Gardnerella* or *Bacteroides-Prevotella* cell types
- grade III (bacterial vaginosis): no *Lactobacillus* cell types, only *Gardnerella*, *Bacteroides-Prevotella* or *Mobiluncus* cell types
- grade IV: predominantly Gram positive cocci

4.2.5.2.2. Evaluation of H_2O_2-production

Swabs were smeared on a TMB plus agar plate (Rabe and Hillier 2003) and incubated for 2 days at 37°C in an anaerobic chamber (BugBox, Ruskinn Technology, LedTechno, Heusden-Zolder, Belgium). The TMB plus agar plate was exposed to air after incubation to evaluate the amount of H_2O_2-production. H_2O_2-production appeared as a blue colour and the number of H_2O_2-producing colonies and the colour intensity was used to score the H_2O_2-production (0: no H_2O_2-production, 1: poor H_2O_2-production, 2: strong H_2O_2-production and 3: very strong H_2O_2-production).

4.3. Results and discussion

4.3.1. In vivo and in vitro evaluation of the distribution and retention of different pellet and powder formulations

Pellets, packed in HPMC or hard gelatin capsules, were evaluated as a new vaginal drug delivery system. The capsule disintegration time, spreading and retention of the different pellet formulations (non-disintegrating MCC pellets versus disintegrating starch-based pellets) and patient acceptability of this dosage form for vaginal application were assessed.

HPMC capsules filled with pellets (MCC or starch-based pellets) adhered well to the vaginal mucosa as no capsules were lost (Groups 1-3, n=15). Although HPMC capsules disintegrated *in vitro* within 10 minutes, *in vivo* disintegration was much slower as even after 6 h 3 out of 5 capsules (Group 2) were still intact. The lack of correlation between *in vitro* and *in vivo* results was probably due to the very low amount of vaginal fluid. However, the capsule wall was weakened as even the slightest touch with the speculum during colposcopy opened the wetted capsules. 24 h after administration all capsules had opened and no capsule remnants were detected. In case of powder-filled HPMC capsules, all capsules (Group 4, n=5) had disintegrated 6 h after administration. Although *in vitro* disintegration was faster for hard gelatin capsules compared to HPMC capsules (3 versus 10 min), their *in vivo* behaviour was similar to that of HPMC capsules as 2 out of 5 (Group 5) were still intact 6 h after administration. This slow capsule disintegration would affect the drug release rate, but this disadvantage could be eliminated if the pellets were administered in the vaginal cavity using an applicator with a different design which does not require that the pellets are packed in a capsule.

In those volunteers where the HPMC capsule (filled with non-disintegrating MCC pellets) (Group 1) had already disintegrated 3 h after administration, pellets clustered around the fornix and no spreading over the lateral walls to the introitus occurred (Fig. 1a). After 24 h a limited number of pellets (< 50) were located at the midportion of the vagina and around the introitus (Fig. 1b) as the volunteers reported that the majority of the pellets was discharged between 5-7 h after administration, mainly during toilet visit and showering. Chatterton et al. (2004) also reported the most loss of a vaginal cream formulation during urination. Hence, despite their small particle size and spherical shape, pellets did not spread evenly and vaginal retention was limited for MCC pellets.

a b

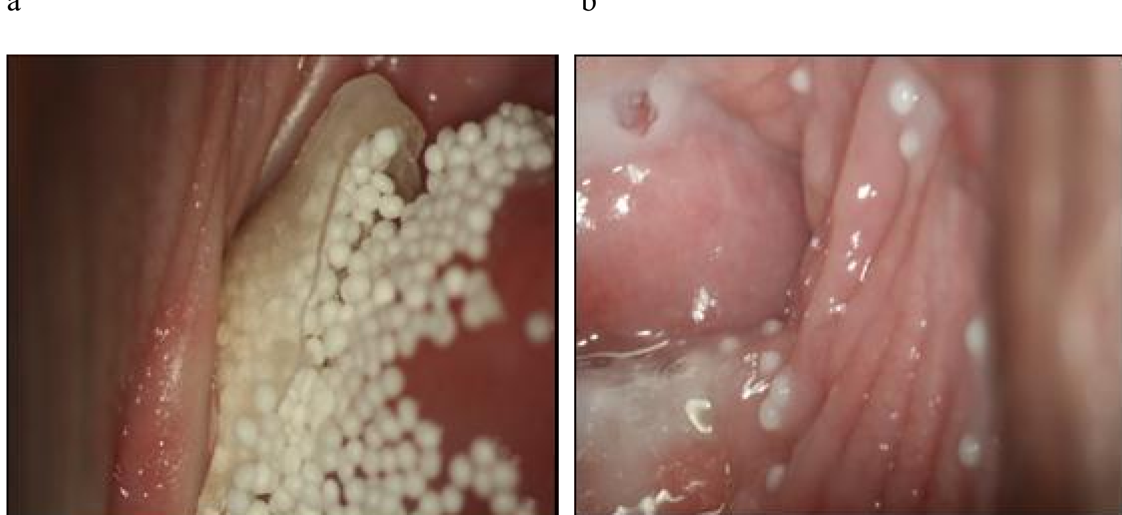

Figure 1 Right side of fornix 3 h (a) and left side of the mid vagina 24 h (b) after administration of an HPMC capsule, filled with non-disintegrating MCC pellets

Although starch-based pellets also clustered around the fornix 6 h after administration, the pellets already started to disintegrate and spreading of the pellets was observed (Fig. 2a) (Group 2). After 24 h for 8 out of 10 volunteers (Group 2-3) complete coverage of the vaginal mucosa with disintegrating pellets was observed (Fig. 2b) as visualised via RSP as a marker.

a b

Figure 2 The fornix anterior 6 h (a) and anterior side of the mid vagina 24 h (b) after administration of an HPMC capsule, filled with disintegrating starch-based pellets

Vaginal swabbing showed that after 6 h, RSP was almost only detected around the fornix, but after 24 h RSP was recovered from all swabbed areas, although the highest concentration was still detected around the fornix (Table 2). In relation to the total amount of RSP administered, the amount recovered via swabbing was low, partly because a fraction of the RSP was already discharged from the vagina, but mainly because only a limited area of the vaginal mucosa was swabbed. In several volunteers no RSP was detected on the swabs taken after 6 h as in these patients the capsules had not yet opened.

Table 2 % RSP of the administered dose detected by swabbing at the fornix, mid vagina and introitus 6 and 24 h after vaginal delivery of an HPMC capsule filled with starch-based pellets (Group 2) or freeze-dried powder (Group 4), with RSP as marker substance

	6 h				**24 h**			
	Fornix	**Mid vagina**	**Introitus**	**Total**	**Fornix**	**Mid vagina**	**Introitus**	**Total**
	Starch-based pellets							
Volunteer 1	0.14	0.01	0.00	**0.15**	0.70	0.25	0.04	**0.99**
Volunteer 2	-	-	-	**-**	0.49	0.10	0.10	**0.69**
Volunteer 3	0.60	0.00	0.00	**0.60**	0.15	0.02	0.00	**0.17**
Volunteer 4	-	-	-	**-**	0.14	0.01	0.00	**0.15**
Volunteer 5	0.48	0.02	0.00	**0.50**	0.34	0.13	0.02	**0.49**
Mean	**0.41**	**0.01**	**0.00**	**0.25**	**0.37**	**0.10**	**0.03**	**0.50**
	Freeze-dried powder							
Volunteer 1	0.30	0.05	0.00	**0.35**	0.07	0.01	0.00	**0.08**
Volunteer 2	0.04	0.00	0.00	**0.04**	0.00	0.00	0.00	**0.00**
Volunteer 3	0.47	0.15	0.17	**0.79**	0.05	0.02	0.00	**0.07**
Volunteer 4	0.01	0.04	0.02	**0.07**	0.03	0.03	0.00	**0.06**
Volunteer 5	0.82	0.00	0.00	**0.82**	0.29	0.08	0.00	**0.37**
Mean	**0.33**	**0.05**	**0.04**	**0.41**	**0.09**	**0.03**	**0.00**	**0.12**

- : capsule still intact 6 h after vaginal administration

After 6 h the powder formulation was mainly spread around the fornix, but already some distribution to the middle anterior and posterior wall of the mid vagina was observed (Table 2) (Fig. 3a) (Group 4). No coverage of the side walls of the vagina midportion was seen. In one volunteer the powder had formed a plug and no spreading occurred. Colposcopy after 24 h revealed only a small amount of powder (n=5) at the posterior and anterior vaginal walls (Fig. 3b), indicating that the powder was faster cleared from the vagina in comparison to pellets. After dispersion of the powder it behaved like liquid and was rapidly cleared from the

vagina. In one volunteer, no powder was detected, whereas another volunteer showed complete vaginal coverage after 24 h.

a b

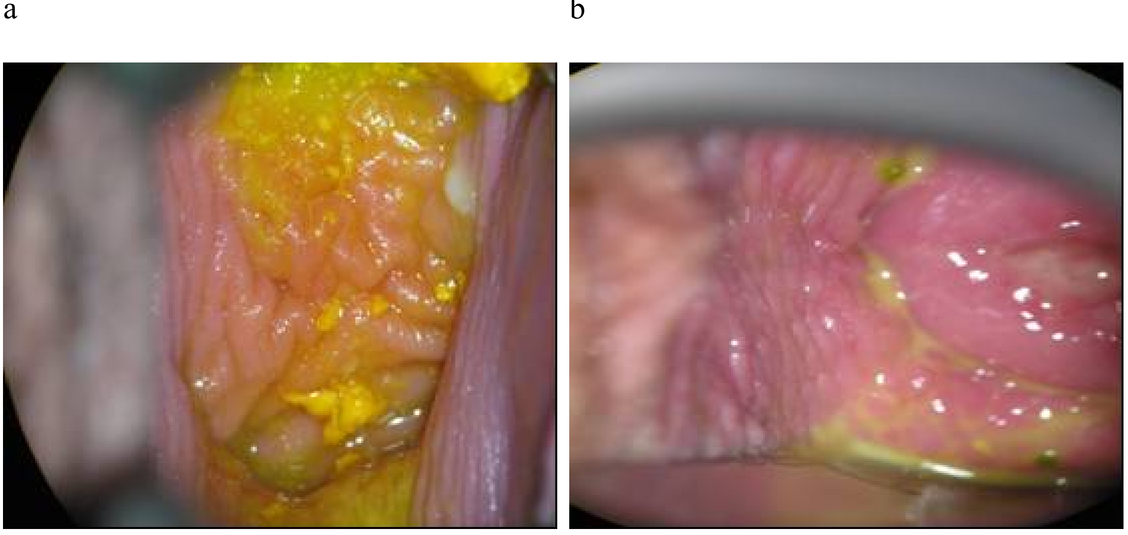

Figure 3 The anterior side of the mid vagina 6 h (a) and right side of the mid vagina 24 h (b) after administration of an HPMC capsule, filled with powder

To evaluate the vaginal clearance of starch-based pellets compared to the powder formulation, pellets and powder discharged from the vagina were collected on panty shields. However, since most pellets and powder were lost during toilet visit and showering, these data did not provide meaningful information.

During the clinical trials only minor side effects were reported by the volunteers (Groups 1-5, n=25): an irritated feeling by one woman and minor low belly pain shortly after administration of the capsule by 3 volunteers. As there was no control group and none of the side effects were severe, they could not be conclusively associated with product use.

4.3.2. In vivo evaluation of the vaginal pH and vaginal flora after pellet and powder administration

If starch-based pellets or lactose/skim milk powder are used as carrier for vaginal probiotic bacteria, it is important to assess the influence of the carrier itself on the vaginal pH and microflora. A control group was included to follow the natural evolution of pH and microflora during a menstrual cycle. Table 3 and Figure 4 shows the average vaginal pH and H_2O_2-scores and Table 4 shows the Gram-staining results in the three groups (control, pellets and powder) at screening, day 6 (one day after the 5-day administration period), day 14, day 22 (after menstruation) and day 35. Only volunteers with normal microflora were included in the study. pH at screening was for all but two volunteers lower than 4.7, which is considered as a normal vaginal pH (range 3.5-4.7). At day 6 (just after administration) and day 14, pH and microflora were normal for all groups. At day 22, just after menstruation, mean pH increased in all groups, however this was due to the period in the menstrual cycle (presence of menstrual flow) and not due to treatment. At day 35 only one person showed a vaginal pH above 4.7.

Table 3 pH and H_2O_2-score (mean ± SD) in control, starch-based pellets and freeze-dried powder group

	Screening	**Day 6**	**Day 14**	**Day 22**	**Day 35**
	pH				
Control group	4.3 ± 0.9	3.8 ± 0.3	3.7 ± 0.2	4.8 ± 1.4	4.0 ± 0.2
Starch-based pellets	4.5 ± 0.9	4.3 ± 0.5	3.9 ± 0.3	5.0 ± 0.8	4.1 ± 0.3
Freeze-dried powder	4.0 ± 0.4	3.8 ± 0.3	3.7 ± 0.2	4.5 ± 0.8	4.3 ± 0.6
	H_2O_2-score				
Control group	2.0 ± 1.3	2.3 ± 1.2	2.3 ± 1.2	0.8 ± 0.8	1.7 ± 1.0
Starch-based pellets	2.0 ± 0.6	2.0 ± 0.9	2.5 ± 0.8	1.3 ± 0.8	1.8 ± 0.4
Freeze-dried powder	1.7 ± 1.0	2.0 ± 1.1	1.7 ± 0.8	0.7 ± 0.5	1.5 ± 0.8

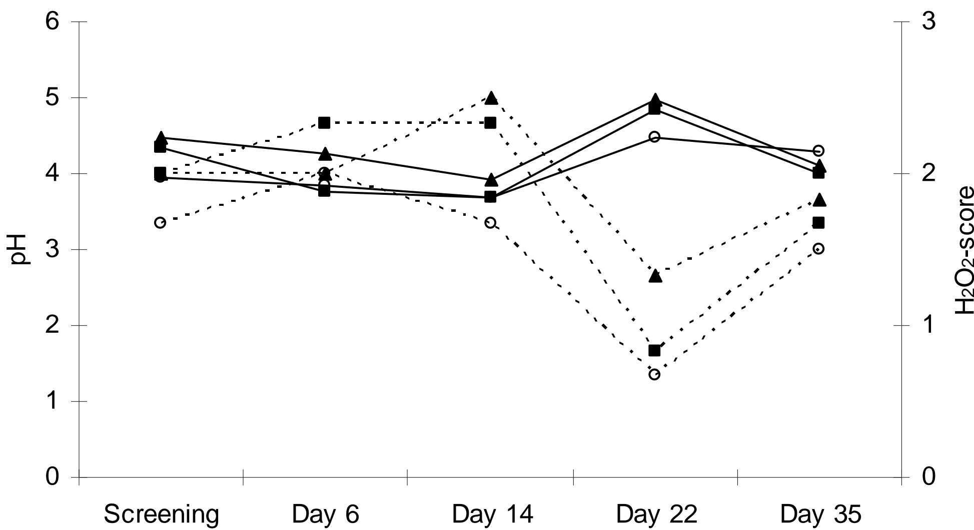

Figure 4 Mean pH (—) and H_2O_2-score (---) in control (■), starch-based pellets (▲) and freeze-dried powder (○) group

Just after menstruation (day 22) 5 abnormal microflora was detected (Table 4), however, again this phenomenon was only due to menstrual bleeding and was not related to treatment (Keane et al. 1997, Eschenbach et al. 2000). Moreover, 3 of the 5 abnormal microfloras were detected in the control group. At day 35, 2 abnormal microflora was detected in the pellet group, however as this abnormal microflora only appears at day 22 and day 35, it was concluded that this change in microflora was not caused by the administered pellet formulation. H_2O_2-production scores of the vaginal microflora at screening were comparable with the production scores on day 35 (Table 3, Fig. 4). On day 22 there was a decrease in H_2O_2-scores in all groups, indicating that just after menstruation there is a decrease in H_2O_2-producing strains (Keane et al. 1997, Eschenbach et al. 2000). H_2O_2-producing vaginal strains are important for a healthy vaginal microflora as the produced H_2O_2 is bactericidal for bacterial vaginosis associated microorganisms.

Microflora and pH at screening and day 35 were similar for all study groups, indicating that starch-based pellets and freeze-dried lactose/skim milk are acceptable carrier materials for the vaginal delivery of probiotic bacteria or other drugs.

Table 4 Gram-staining results in control, starch-based pellets and freeze-dried powder group

	Screening	Day 6	Day 14	Day 22	Day 35
	Control group				
Volunteer 1	Ia	Ia	Ia	Ia	Ia
Volunteer 2	Ia	Ia	Ia	Ia	Ia
Volunteer 3	Ia	Ia	Ia	Ia	Ia
Volunteer 4	Ib	Ia	Ia	II	Ia
Volunteer 5	Iab	Ib	Ia or b	III	Ia or b
Volunteer 6	Ia	Ia	Ia	II	Ia
	Starch-based pellets				
Volunteer 1	Ib	Ib	Ib/I-like	I-like	I-like
Volunteer 2	Ia	Ia	Ia	Iab	Ia
Volunteer 3	Ib	Ib	Ib	Ib	Ib
Volunteer 4	Ia	Ia	Ia	/	Ia
Volunteer 5	Ia	Ia	Ia	Iab	Ia(b)
Volunteer 6	Ib	Ib	Ib	Ib	III
	Freeze-dried powder				
Volunteer 1	Ib	Ib	Iab	Iab	Ia(b)
Volunteer 2	Ib	Ib	Ib + a	Ia	Ib
Volunteer 3	Ia	Ia	Ia	Ia	Ia
Volunteer 4	Ia	Ia	Ia	Ia	Ia
Volunteer 5	Ia	Ia	Iab	Ia	Ia
Volunteer 6	Ia	Ia	Ia	Ib	Iab

Red: abnormal microflora

4.4. Conclusion

Disintegrating starch-based pellets have been identified as a promising novel vaginal drug delivery system, which resulted in complete coverage of the vaginal epithelium. A drawback of this formulation was the slow disintegration of the capsules, however using an applicator that does not require to pack the pellets in capsules prior to vaginal delivery would eliminate this problem. These starch-based pellets are acceptable carriers for probiotic bacteria or other drugs, as the carrier itself had no influence on vaginal pH and microflora.

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5

DEVELOPMENT OF A VAGINAL FORMULATION OF *LACTOBACILLUS* SPECIES AND *IN VIVO* EVALUATION IN HEALTHY VOLUNTEERS

5.1. Introduction

Bacterial vaginosis and yeast vaginitis are affecting a large number of women and these ailments are major public health concerns. Generally within these patients the normal vaginal microflora is disturbed and the natural protection against infectious microorganisms is lost. The use of probiotic microorganisms to restore the normal vaginal flora is gaining interest as an alternative to conventional antibiotic treatment (Andreu 2004; Falagas et al. 2007; Famularo et al. 2001; Hoesl and Altwein 2005; Reid and Bruce 2003). Four vaginal strains have been identified at the Laboratory for Bacteriology Research (Ghent University Hospital) (Saerens 2006) as potential probiotics to treat bacterial vaginosis, i.e. *Lactobacillus jensenii* PB204-T1-1, *Lactobacillus crispatus* PB125-T1-1, *Lactobacillus crispatus* PB128-T1-1 and *Lactobacillus gasseri* PB088-T2-1. These strains were selected based on their (1) capacity to

produce H_2O_2, (2) inhibition of *Gardnerella vaginalis, Atopobium vaginae* and uropathogenic *Escherichia coli*, (3) absence of bacteriophage production, (4) absence of growth inhibition of other *Lactobacillus* species.

However, to efficiently deliver these probiotics to the vagina these strains must be formulated in a dosage form that maintains the viability of these microorganisms during manufacturing and shelf life. In addition the carrier formulation of the probiotics must ensure that residence time and spreading of viable microorganisms in the vaginal cavity is sufficient to colonize the vagina and restore the microflora. Therefore this study evaluates the viability of the selected lactobacilli strains in function of the manufacturing technique (freeze-drying versus layering on inert carriers) using different dosage forms (layered pellets, powder) and excipients (lactose, skim milk, trehalose, vitamin C) to maximize viability. The benefits of tangential flow filtration compared to centrifugation for harvesting the bacteria from the culture medium were also evaluated. Finally a clinical trial in healthy volunteers was performed to compare the vaginal colonisation potential of 3 probiotic strains, administered via layered pellets or freeze-dried powder.

5.2. Materials and methods

5.2.1. Materials

Growth medium MRS was purchased from Oxoid (Cambridge, UK). Tryptic Soy Agar (TSA) plates, enriched with 5 % sheep blood, were obtained from Becton Dickinson (Becton Dickinson, Franklin Lakes, NJ, USA). Microcrystalline cellulose pellets were used as inert carriers for the layering experiments (Cellets® 500 µm, Pharmatrans-Sanaq, Basel, Switzerland). Lactose (α-Pharma, Braine-l'Alleud, Belgium), skim milk (Difco, Becton

Dickinson, MA, USA), trehalose (Cerestar, Mechelen, Belgium) and vitamin C (Certa, Braine-l'Alleud, Belgium) were used as stabilisers. During the *in vivo* study starch-based pellets consisting 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, New Jersey, USA) were used as carriers for the lactobacilli.

5.2.2. Bacterial strains

Lactobacillus jensenii PB2003/204-T1-1, *Lactobacillus gasseri* PB2003/088-T2-1, *Lactobacillus crispatus* PB2003/125-T1-1 and *Lactobacillus crispatus* PB2003/128-T1-1 were obtained from the Laboratory for Bacteriology Research (Ghent University Hospital). A stock of each individual *Lactobacillus* strain was stored at -70°C in TSB (Tryptic Soy Broth) with 15 % glycerol.

5.2.3. Preparation of the layering and freeze-drying suspensions

Bacteria were inoculated from the stock solution on a TSA plate enriched with 5 % sheep blood and incubated for 48 h at 37°C under anaerobic conditions (BugBox, Ruskinn Technology, LedTechno, Heusden-Zolder, Belgium). After 48 h one colony was isolated and inoculated in 5 ml MRS broth under anaerobic conditions for 24 h (overnight culture), where after a preculture of 100 ml was inoculated with 1 ml of the overnight culture and incubated for another 24 h. 10 L of *Lactobacillus* culture was prepared by inoculating 10 flasks with 1 L MRS broth with 10 ml of the preculture. The 10 L cultures were grown until the stationary phase was reached, i.e. 17 h for *L. jensenii*, 48 h for *L. gasseri* and *L. crispatus* 128 and 72 h

for *L. crispatus* 125. For *L. crispatus* 125 an MRS-acetate broth (pH 6) was used. In one experiment the growth medium was enriched with 6 g/L trehalose as additional carbon source. Afterwards the bacteria were collected by centrifugation (Beckmann JA10 rotor, Munchen, Germany). The centrifugation parameters are summarised in Table 1.

Table 1 Centrifugation parameters used to harvest the bacteria from the growth medium

	g	**Time (min)**	**Temperature (°C)**
L. jensenii	2800	15	4
L. gasseri	3100	20	4
L. crispatus 125	2800	20	4
L. crispatus 128	3100	20	4

To obtain a 100-fold concentrated bacterial suspension the cell pellet harvested from a 10 L culture was resuspended in 100 ml of a 10 % lactose/20 % skim milk (w/v) solution which was used as stabilising matrix to maintain the viability of the lactobacilli strains during processing. To prevent further activity or growth, the cultures were kept on ice during all handling steps. In additional experiments 1 % vitamin C was added to the stabilising matrix or the lactose fraction was substituted by trehalose.

In addition a 50 L culture of *L. crispatus* 128 was grown using a U50 Bioreactor (Sartorius BBI systems, Goettingen, Germany) at the Laboratory of Industrial Microbiology and Biocatalysis (Faculty of Bioscience Engineering, Ghent University). A pre-culture of 5 ml MRS broth was inoculated in 500 ml MRS broth. After 26.5 h at 37°C a mid-exponential culture was obtained and inoculated in the 50 L fermentor. Fermentation was performed at a rotational speed of 150 rpm without aeration and after 25 h the stationary phase was reached. After 40 h the culture was centrifuged (Cepa-centrifuge, Lahr, Germany) for 45 min at a rotation speed of 17670*g*.

5.2.4. Layering process

As the inert carrier had no influence on the viability of *Lactococcus lactis* after layering (see Chapter 2), MCC pellets (Cellets® 500 µm) were used as inert carriers to assess the viability of the lactobacilli. Layering was performed using the protocol described in Chapter 2. After layering pellets were packed in Alu sachets (LPS, Vapor flex barrier bag, NJ, US) and stored for up to 1 year at -20 and 4°C and at 20 % relative humidity.

5.2.5. Freeze-drying

Petri dishes (diameter: 85 mm) filled with 20 ml of the bacterial suspension were freeze-dried using an Amsco Finn Aqua GT4 freeze-dryer (Amsco, Germany). The dispersion was frozen to -45°C within 175 min at 1000 mbar. Primary drying was performed at -15°C and at a pressure varying between 0.8 and 1 mbar during 13 h and was followed by secondary drying at elevated temperature (10°C) and reduced pressure (0.1–0.2 mbar) for 7 h. The obtained cake was crushed with mortar and pestle, packed in Alu sachets and stored for up to 1 year at -20 or 4°C and a relative humidity of 20 %.

5.2.6. Harvesting of bacteria by tangential flow filtration

Tangential flow filtration was performed by means of a Proflux M12 system (Millipore, Bellerica, MA, USA) using a 0.45 µm polyvinylidene fluoride membrane (Durapore V-screen Pellicon 2 cassettes) to isolate the microorganisms (Fig. 1). 2 L stationary phase cultures were prepared of the four *Lactobacillus* strains. The culture was separately added to the reservoir compartment and concentrated with a permeate flow of about 115 ml/min until the minimum

working volume of the M12 Proflux system was reached (about 110 ml). The retentate was collected and the filter unit flushed with 200 ml fresh MRS to collect the bacteria retained on the membrane. The concentrated culture and the MRS flush medium were centrifuged at 3000*g* for 20 min to remove the residual growth medium. The cell pellet was resuspended in 20 ml stabilising matrix (10 % lactose/20 % skim milk, w/v) and freeze-dried as described above. To compare the different techniques used to harvest lactobacilli from the growth medium (centrifugation vs. tangential flow filtration) a 2 L stationary phase culture was simultaneously processed by centrifugation and the resulting cell pellet was also freeze-dried after resuspending in 20 ml stabilising matrix. Viability of the microorganisms in the stationary phase culture, the MRS flush, the stabilising matrix and the freeze-dried powder was assessed.

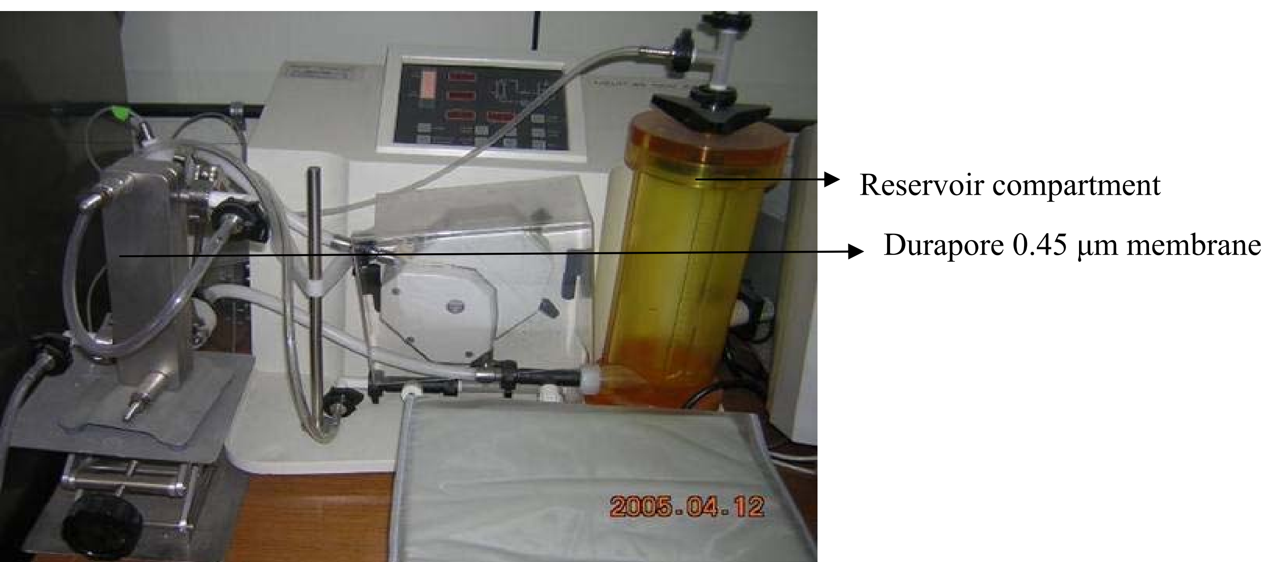

Figure 1 The proflux M12 system

5.2.7. Viability assessment

Viability of layered and freeze-dried bacteria was assessed immediately after drying (via layering or freeze drying) and after storage (at -20 or 4°C). 100 mg of the formulation was resuspended in 1 ml MRS broth and a dilution series was plated out. For *L. jensenii* and *L. crispatus* 128 a plate pour method was used with MRS agar as growth medium. For *L. gasseri* and *L. crispatus* 125 a plate spread method was used with TSA, enriched with 5 % sheep blood. After 48 h anaerobic incubation (BugBox, Ruskinn Technology, Pencoed, UK) at 37°C the colony forming units were counted.

5.2.8. In vivo evaluation of the colonisation potential of a pellet and powder formulation containing Lactobacillus species

Hard gelatin capsules were filled with 5×10^6 *L. jensenii*, *L. crispatus* 125 and *L. crispatus* 128 formulated as a pellet or powder formulation. The pellet formulation consisted of starch-based pellets layered with bacteria using 10 % lactose/20 % skim milk as stabilising matrix. The powder formulation consisted of bacteria stabilised in 10 % lactose/20 % skim milk. A double-blind, randomised, parallel study was performed with 16 healthy volunteers. 8 women followed a 5-day treatment with pellet-filled capsules, 8 women followed a 5-day treatment with powder-filled capsules. The same restrictions were applied as in the *in vivo* study with the placebo formulations (see Chapter 4).

On day 1, 2, 3, 4 and 5 one capsule was administered by the volunteers just before bedtime using a vaginal applicator. On day 22 the intake of the monophasic pill was stopped.

On day 6, 9, 12 and 33 (eleven days after pill stop) the volunteer underwent a gynaecological examination. The vagina and rectum were swabbed with an Eswab (Copan diagnostics,

Corona, CA, USA). Swabs were used for Gram-staining and culturing (to identify H_2O_2-producing strains) (using the method described in Chapter 4) at the Laboratory for Bacteriological Research (Ghent University Hospital).

5.3. Results and discussion

5.3.1. Viability after layering and freeze-drying

The survival data of the formulations after layering and freeze-drying are summarised in Table 2 and 3, respectively. The viability immediately after drying was expressed as percentage survival i.e. the ratio of viable bacteria after drying to the number of bacteria in the suspension used for drying. The viabilities after storage are expressed as relative viabilities (%) i.e. the ratio of viable bacteria after storage to the number of bacteria immediately after drying. 10 % lactose/20 % skim milk was selected as stabilising matrix as this combination showed the best stabilising capacities for a *L. lactis* strain (Poelvoorde et al. 2008).

For all strains freeze-drying resulted in higher survival ratios immediately after drying compared to layering. *L. gasseri* could not be processed via layering as the pellets agglomerated during processing due to the sticky nature of the *L. gasseri*-containing matrix. However the layered formulation of *L. jensenii* and *L. crispatus* 128 offered the best stability during storage. These results confirmed that survival after drying was strain dependent (Andersen et al. 1999; Carvalho et al. 2004b; Champagne et al. 1991; To and Etzel 1997) and that long term storage - even at low temperature- resulted in a large decrease of viability.

Table 2 Viability (%) of the *Lactobacillus* strains after layering on inert microcrystalline cellulose carries and storage at 4°C

	L. jensenii	***L. crispatus* 125**	***L. crispatus* 128**	***L. gasseri***
After layering[a]	5.57	1.73	10.91	-
1 month storage at 4°C[b]	14.55	28.06	50.96	-
9 months storage at 4°C[b]	0.34	1.78	16.93	-

[a] the percentage survival immediately after drying is expressed as the ratio of viable bacteria after drying to the number of bacteria in the suspension used for drying, [b] the percentage survival after storage is expressed as the ratio of viable bacteria after storage to the number of bacteria immediately after drying

Table 3 Viability (%) of the *Lactobacillus* strains after freeze-drying and storage at 4°C

	L. jensenii	***L. crispatus* 125**	***L. crispatus* 128**	***L. gasseri***
After freeze-drying[a]	27.44	6.02	13.14	35.34
1 month storage at 4°C[b]	0.12	55.13	2.37	5.93
9 months storage at 4°C[b]	0.03	18.39	0.02	0.07

[a] the percentage survival immediately after drying is expressed as the ratio of viable bacteria after drying to the number of bacteria in the suspension used for drying, [b] the percentage survival after storage is expressed as the ratio of viable bacteria after storage to the number of bacteria immediately after drying

For vaginal delivery pellets and powder were packed in size 00 capsules and vaginally administered using a commercially available applicator (as described in Chapter 4). About 750 mg pellets or 400 mg freeze-dried powder can be packed in one capsule and the number of bacteria per capsule immediately after drying and after 9 months storage is presented in Table 4. Although immediately after drying a high dose of bacteria per capsule was obtained, the dose decreased to about 10^6 cfu per capsule after 9 months storage at 4°C. Although a dose finding study of Reid et al. (2001) for vaginal colonisation via the oral route concluded that an oral dose of 10^8 cfu was sufficient to obtain vaginal colonisation, the required dose for vaginal administration is unknown. However, based on the local delivery via capsules and the

direct colonisation of the lactobacilli one can expect that a lower dose is required for the vaginal route compared to oral administration of lactobacilli.

Similar stability data were obtained by Maggi et al. (2000): a 3 log decrease of viability for an freeze-dried *L. crispatus* strain and a 2 log reduction for a freeze-dried *L. gasseri* strain after 1 year storage at 4°C using a combination of skim milk and malt extract as stabilising matrix. Otero et al. (2007) combined skim milk, lactose and sucrose with the highest survival ratio for a 6 % skim milk/6 % lactose-mixture: 9.00 log cfu/g for *L. gasseri* CRL1412 and 8.84 log cfu/g for *L. gasseri* CRL 1421 after 9 months storage at 2-8°C when the freeze-dried powder was packed in sealed ampoules. In contrast, packing of the freeze-dried powder in gelatin capsules reduced the viability after 9 months to 8.62 and 7.88 log cfu/g for *L. gasseri* CRL 1412 and 1421, respectively.

Table 4 Viability of the *Lactobacillus* strains after drying via layering and freeze-drying and after 9 months storage at 4°C. The viability is expressed as the number of colony forming units (cfu) per size 00 capsule.

	L. jensenii	***L. crispatus* 125**	***L. crispatus* 128**	***L. gasseri***
After layering	7.85×10^{8}	9.12×10^{7}	3.42×10^{8}	-
9 months storage at 4°C	2.69×10^{6}	1.63×10^{6}	5.15×10^{7}	-
After freeze-drying	2.27×10^{10}	1.86×10^{9}	2.42×10^{9}	5.96×10^{9}
9 months storage at 4°C	6.25×10^{6}	3.42×10^{8}	3.68×10^{5}	4.29×10^{6}

Although hard gelatin capsules would be preferred over HPMC capsules for vaginal delivery of the probiotics based on their slightly faster vaginal disintegration compared to HPMC capsules (Chapter 4), the higher water content of the hard gelatin capsule shells (13-15% vs. 2-3% for HMPMC capsules) could have a detrimental effect on storage stability of the microorganisms. Therefore pellets layered with *L. jensenii* and a freeze-dried formulation of *L. jensenii* were packed into hard gelatin as well as HPMC capsules (which are both

commonly used in the pharmaceutical industry) and the viability was monitored over a 9 month period during storage at 4°C (Table 5). Although the 9 months viability had decreased in all cases (independent of formulation and packaging), there was no difference in viability of *L. jensenii* stored in HPMC or gelatin capsules. Hence the higher water content of the gelatin capsules did not affect bacterial survival during storage.

Table 5 Viability of *L. jensenii* after drying via layering and freeze-drying and after 9 months storage at 4°C (relative humidity of 20%) in gelatin or HPMC capsule. The viability is expressed as the number of colony forming units (cfu) per size 00 capsule.

	Gelatin capsules	**HPMC capsules**
After layering	3.41×10^8	3.41×10^8
9 months storage at 4°C	2.92×10^6	1.39×10^6
After freeze-drying	8.70×10^8	8.70×10^8
9 months storage at 4°C	3.77×10^6	4.72×10^6

Despite the high load after processing a higher cell load is preferred to assure colonisation and long term storage stability (>9 months). To improve bacterial survival after drying and storage different approaches were used: (1) increasing the cell load of the drying suspension and (2) modification of the layering matrix. Via optimisation of the growth conditions of the different strains it was attempted to achieve a higher cell load of the drying suspension. The atmospheric conditions (aerobic, anaerobic, under CO_2), the growth conditions (with or without shaking) and the growth medium (phosphate, acetate and carbonate buffered growth media) were included as variables. However, independent of the conditions the cell concentration in the medium could not be improved. As increasing the bacterial cell concentration in the stationary phase culture was not possible by changing the incubation conditions, a 50 L fermentation of *L. crispatus* 128 was performed to obtain a higher concentration factor after resuspending the cell pellet (500-fold vs. 100-fold). However, after

this large scale fermentation a cell pellet of 507 g was obtained, which could not be resuspended in 100 ml of stabilising matrix used for the layering experiment.

In a second approach the stabilising matrix was modified to achieve a higher survival rate. Ascorbic acid was added to the stabilising matrix as vitamin C has an anti-oxidant function which can be beneficial during storage of dried bacteria by delaying auto-oxidation of membrane phospholipids (Carvalho et al. 2004b). *L. jensenii* was selected as test strain. Viability after storage improved as 1 % vitamin C was added to a 10 % lactose/20 % skim milk matrix for layering of the *L. jensenii* strain (using a 1 L overnight culture which was 10-fold concentrated in the stabilising matrix): viability immediately after layering was 13.01 % and the survival rate after 12 months storage at 4°C improved from 5.40 % without vitamin C to 68.14% in combination with vitamin C (Fig. 2). Zarate and Nader-Macias (2006) also reported stability improvement after freeze-drying of different *Lactobacillus* strains if vitamin C was added to lactose/skim milk matrices.

Lactose was substituted by trehalose as stabilising disaccharide as special stabilising capacities are attributed to trehalose based on its higher T_g compared to other dissaccharides, which is maintained if small amounts of water were added (Crowe et al. 1996). However, incorporation of trehalose in the formulation did not improve stability (Fig. 2).

In another experiment trehalose was added to the growth medium of the *L. jensenii* strain as a growth medium containing other sugar substrates produces cells with distinct morphological and physiological characteristics, which can be reflected in their resistance to stress conditions (Carvalho et al. 2004a; Carvalho et al. 2004b). A 10 % lactose/20 % skim milk mixture was used as external stabilising matrix. However, after addition of trehalose to the growth medium similar survival rates were obtained after drying or storage (Fig. 2). Moreover the carriers tended to agglomerate during layering, possibly due to the production of exopolysaccharide (EPS) by the bacteria as this depends on the carbon source present in the

growth medium (Degeest et al. 2001). However, no correlation was found between EPS production and survival after freeze-drying or during storage of freeze-dried bacteria (Carvalho et al. 2004a; Looijesteijn et al. 2001).

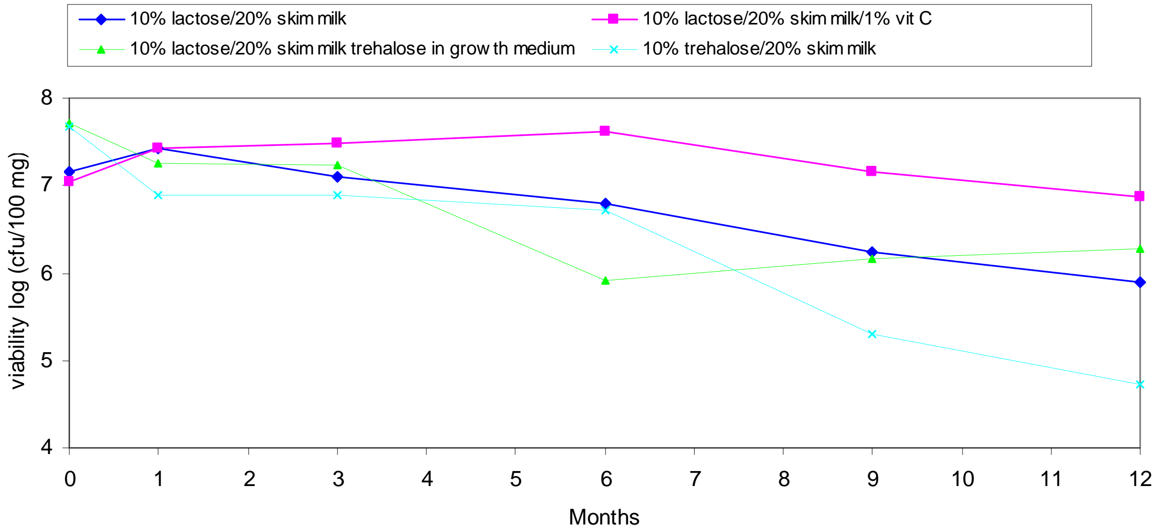

Figure 2 Viability (cfu/100mg) of *L. jensenii* after layering and storage using different stabilising matrices

5.3.2. Tangential flow filtration

Centrifugation is generally used to harvest bacteria from a liquid culture. However, centrifugation is a time consuming process and it can damage the bacteria due to the large forces generated during centrifugation. In addition up-scaling of the centrifugation process is troublesome. As alternative harvesting technique tangential flow filtration was evaluated (Naja et al. 2006; Sundaran et al. 2002). Tangential flow filtration is a pressure-driven membrane separation process, which provides a separation based on the molecular size of the constituents. Soluble and low molecular weight materials pass through the membrane and bacteria which are larger than the membrane pore size are retained. The feed is pumped across the membrane surfaces and the pressure gradient through the membrane acts as a driving

force to push the solutes and the water through the membrane. Up-scaling is easier and tangential flow filtration can be seen as a continuous process, because feed can be continuously added during the process as permeate is removed. A drawback of tangential flow filtration is that not all the growth medium is removed during filtration, which is not acceptable for our application as residues of the MRS-growth medium induce sticking problems during layering. As a consequence an additional centrifugation step was required to maximize removal of the growth medium before resuspending the cell pellet in a solution of stabilising sugar.

Bacterial suspensions containing the different *Lactobacillus* species were processed via centrifugation and tangential flow filtration to assess the cell load obtained via both processes as well as the effect of this pre-processing step on the survival rate after freeze-drying. The concentration of bacteria in the stabilising matrix after centrifugation and microfiltration and the viability after freeze-drying is shown in Figure 3 and 4, respectively. For all bacteria the concentration in the stabilising matrix was comparable after centrifugation and tangential flow filtration. Since a large fraction of bacteria (between 16.9 and 35.3 %) adhered to the membrane, an additional flushing step was required to obtain a high yield of the tangential filtration process.

If these stabilising matrices were freeze-dried, similar viability data were obtained for both concentrating procedures, indicating that there was no influence of the harvesting technique on the ability to withstand freeze-drying. Since centrifugation did not damage the bacteria tangential flow filtration did not offer an added value.

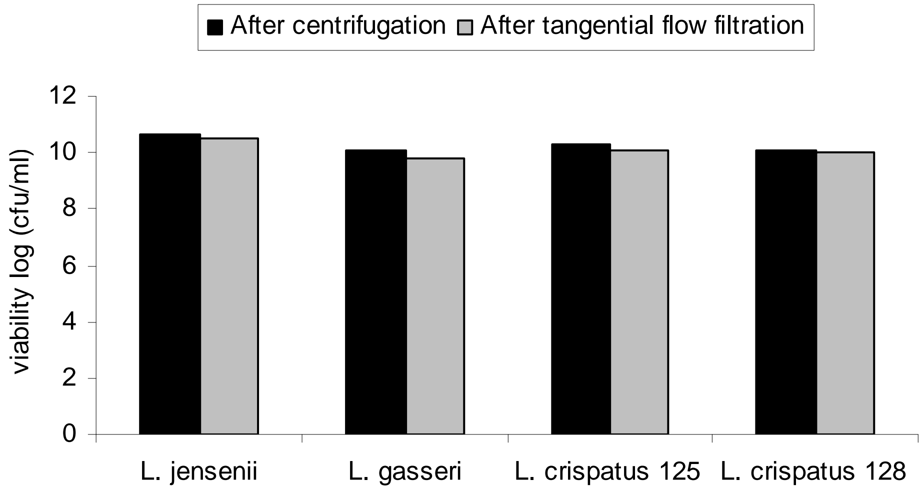

Figure 3 Viability log (cfu/ml) in the stabilising matrix after centrifugation and tangential flow filtration

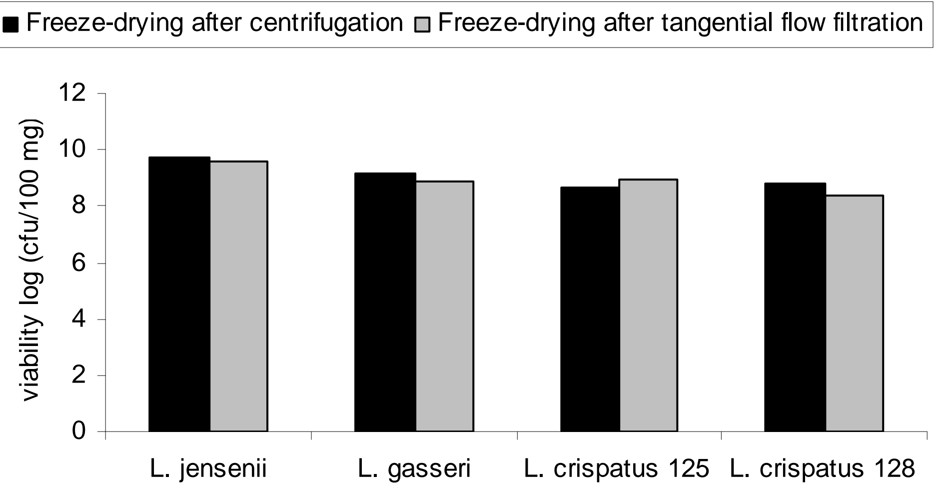

Figure 4 Viability log (cfu/100 mg) in the freeze-dried powder after centrifugation and tangential flow filtration

5.3.3. In vivo colonisation study

During a clinical trial the colonisation potential of the probiotic strains *L. jensenii*, *L. crispatus* 125 and *L. crispatus* 128 was evaluated and the colonising capacity of the pellet and powder formulation (both containing 5×10^6 of each strain) was compared (Table 6). *L. gasseri* was excluded as no layered multi-particulate formulation could be obtained.

At the start of the 5-day treatment *L. jensenii* was detected in two volunteers, *L. crispatus* 128 strain was present in 8 of 16 volunteers and *L. crispatus* 125 was not found at screening. In those subjects that were already colonised at screening by a strain with an RAPD pattern (random amplification of polymorphic DNA) that equals the patterns of the administered probiotic strains, the colonisation potential of that specific strain could not be determined. It was defined that colonisation was achieved if a specific strain was present from day 6 until day 33.

On day 6 (1 day after the end of the treatment) *L. jensenii* was detected in 7 of the 14 volunteers. All but one of these women were still colonised with this strain on day 12 and 33. In three other volunteers *L. jensenii* was only recovered on one or two check-up days, indicating only a transient colonisation.

L. crispatus 128 colonised just one volunteer from day 6 until day 33. In two other volunteers a temporary colonisation was detected. These low colonisation results can be due to the administered dose (i.e. 5×10^6), which might be too low to allow colonisation with this strain. Similar results were seen for *L. crispatus* 125 since this strain was only found in one subject after treatment.

The rectum was also swabbed to evaluate rectal colonisation with the probiotic strains as the rectum can serve as reservoir for vaginal colonisation. In 8 of the 16 subjects H_2O_2- producing strains were detected in the rectum at one follow-up day and in 4 of these women these strains were identified as the administered probiotic strains. However, rectal colonisation was of short duration as the strains were detected on a single day and not over a longer period.

Of 12 women having a normal vaginal microflora at the onset of the study, one person evolved to bacterial vaginosis (III) at day 33, but one women having bacterial vaginosis at the start evolved to a less disturbed microflora (Ibc) after the study. However, these abnormal microflora did not influence the conclusion from the colonisation study.

As vaginal colonisation was poor for the *L. crispatus* strains and rectum colonisation was only temporary, only the vaginal colonisation of *L. jensenii* was taken in consideration to distinguish between the pellet and powder formulation. From the 6 volunteers that were colonised with *L. jensenii* from day 6 until day 33, three received the pellet formulation and 3 the powder formulation, indicating that the formulation itself did not influence colonisation.

Table 6 Colonisation potential according to Gram-stain results

	Screening	**Day 6**	**Day 9**	**Day 12**	**Day 33**
	Pellet formulation				
Volunteer 1	Ia	Ia	Ia	Ia	Iab
Volunteer 2	Ia	Ia	Ia	Ia	Ia
Volunteer 3	Ia	Ib	Ic	Ic	Ic
Volunteer 4	Ia	Ia	Iab	Ia	Iab
Volunteer 5	Ia	Ia	Iab	Ib	Ia
Volunteer 6	Iab	Ia	Iabc	II	III
Volunteer 7	Iab	Ia	Ia	Ia	Iab
Volunteer 8	Ib	Ia	Iab	Ib	Iab
	Powder formulation				
Volunteer 1	Ia	Ia	Ia	Ia	Iab
Volunteer 2	Iab	Iab	Iabc	Iab	Ia
Volunteer 3	Iac	Iac	Iabc	Ibc	Iabc
Volunteer 4	Ib + yeast	Ib + yeast	Ib + yeast	Ib + yeast	Iab
Volunteer 5	Ic + yeast	Iabc + yeast	III + yeast	III + yeast	III + yeast
Volunteer 6	Ic	Ic	Ic	Ic	III
Volunteer 7	III	III	III	III	Ibc
Volunteer 8	IV	Iac	Iab	Iab	IV

Red: abnormal microflora

Colonised with *L. jensenii* | Colonised with *L. crispatus* 128 | Colonised with *L. crispatus* 125

5.4. Conclusion

The survival rate of four *Lactobacillus* species after drying via freeze-drying or layering was strain dependent. For all strains under investigation freeze-drying resulted in a higher viability immediately after layering. However for 3 of the tested bacteria viability decreased significantly after 1 month storage of the freeze-dried powder, whereas the layered pellets had a slower decrease in viability. The highest stability during storage at 4°C was obtained if vitamin C was added as an anti-oxidant to the layered pellet formulation.

Vaginal colonisation of the *L. jensenii* strain was detected after vaginal administration of layered pellets as well as a powder formulation, but the clinical effect of this colonisation must be confirmed in patients with recurring bacterial vaginosis.

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GENERAL CONCLUSION AND FUTURE PERSPECTIVES

This research work described the formulation of dry, viable bacterial strains, which can be used for their health promoting or therapeutic characteristics. Layering was identified as a suitable processing technique for the development of a dry multi-particulate formulation containing viable genetically modified *Lactococcus lactis,* which can be used for treatment of patients suffering from Crohn's disease. Multi-particulates assure a fast gastric emptying and can be enteric coated in the same process step, which make them more advantageous than coated capsules filled wit freeze- or spray-dried powder. Layering of *L. lactis* on inert carriers using a combination of lactose and skim milk as stabilising matrix resulted in a high load of viable *L. lactis*. However, long term storage without loss of viability was only possible at -20°C, despite the addition of different stabilising molecules to the layered formulation. Although the viability of *L. lactis* decreased during simulated gastric passage of Eudragit® FS30D coated pellets, a sufficient amount of viable IL-10 producing *L. lactis* could be delivered to the ileum, the intended target site. Release targeted to the ileum, the most infected part of the intestine in patients with Crohn's disease, could be achieved using Eudragit® FS30D, if a small coating thickness was used or if the amount of free carboxylic groups on the polymer was increased by basic hydrolysis.

The layered pellet formulation was also suitable for vaginal delivery of probiotic strains (*Lactobacillus jensenii* and *Lactobacillus crispatus*) using starch-based pellets as carrier. These starch-based pellets were well retained and distributed in the vaginal cavity in contrast

to non-disintegrating microcrystalline cellulose pellets and freeze-dried powder. Moreover, starch-based pellets did not influence the natural pH or microflora. Vaginal colonisation was obtained for *L. jensenii* after a 5-day treatment in healthy volunteers using a dose of 5×10^6 cfu. However, future optimisation work is recommended as no colonisation was obtained for the *L. crispatus* strains and the clinical effect in patients with recurrent bacterial vaginosis must be demonstrated. Recently, a clinical trial was started in patients with bacterial vaginosis. Bacterial vaginosis patients will receive a vaginal probiotic formulation containing *L. jensenii* and *L. crispatus* on 5 consecutive days immediately after a traditional metronidazole treatment. One month afterwards patients will receive a second cure with the same dose of probiotic bacteria. This treatment group will be compared with a control group who receives a placebo formulation after antibiotic treatment. The vaginal colonisation with the administered strains and the (re-)occurrence of bacterial vaginosis will be evaluated.

The main issue in the formulation of this type of viable therapeutics, is their viability after drying and storage. As the long term storage stability of all strains evaluated in this research project was poor, additional research is required to optimize the formulation and/or processing technique to improve the long term survival of the microorganisms during storage since a reliable formulation with an acceptable shelf life is an essential aspect for the successful application of bacteria as therapeutics in disease treatment. Addition of vitamin C to the layered formulation of *L. jensenii* resulted in promising stability results, but this must be further explored for the other strains. The use of different growth media and growth conditions and the removal of fermentation products and other by-products of microbial metabolism before drying was not evaluated in the present studies. These (and other optimisations) may increase the survival of the bacteria during drying and storage.

In addition, in case of oral delivery, gastric protection was poor and should be improved to assure that a proper dose reaches the intestine.

Although the potential of a multi-particulate starch-based pellet formulation for vaginal delivery of probiotics was clearly highlighted in this study, the opportunities of this novel vaginal delivery platform for administration of conventional drugs (e.g. microbiocides) needs to be further investigated to identify the full potential of this delivery system. For a proper evaluation of the vaginal behaviour of multi-particulates, a non-invasive evaluation technique should be used, like gamma scintigraphy, which does not interfere with the distribution and retention of the formulation. Moreover, the development of a suitable animal model to evaluate vaginal distribution and retention of pharmaceutical formulations will be of high value, as it is not possible to perform all tests directly on humans. As capsules showed a very slow vaginal disintegration (in some cases more than 6 h), it should be evaluated if another delivery device can be used.

SUMMARY

1 The aim of this research project was to develop multi-particulate dosage forms for delivery of lactic acid bacteria: (a) the development of an oral dosage form containing a high concentration of viable, genetically modified, IL-10 producing *Lactococcus lactis* (b) the development of a formulation for delivery of health-promoting strains of lactic acid bacteria (referred to as probiotics) to the vagina. In addition to the viability of the lactobacilli strains incorporated in the formulation the vaginal retention time and spreading of the formulation in the vaginal cavity are essential quality parameters of this vaginal formulation. Chapter 1 reviews the use of genetically modified *Lactococcus lactis* and probiotic *Lactobacillus* strains as therapeutics in the treatment of Crohn's disease and bacterial vaginosis, respectively. The strategies used to stabilise lactic acid bacteria in solid dosage forms and the processing techniques used to manufacture the oral and vaginal dosage forms are also discussed in this introductory chapter.

2 In Chapter 2 an oral multi-particulate formulation of viable *Lactococcus lactis* Thy 12 was developed using fluidized bed layering on inert carriers. Bacteria were suspended in a stabilising matrix, sprayed and subsequently dried on pellets. A multi-particulate formulation was selected as dosage form as it assures fast gastric emptying of the small subunits of the formulation and after layering of the microorganisms on an inert carrier the formulation can be immediately enteric coated using the same process equipment. Chapter 2.3.1. describes the optimisation of the Bioscreen turbidity method, used for viability assessment of *L. lactis* in the dried samples. In chapter 2.3.2. the layering process was optimised by modification of different process and formulation parameters. Atomising

pressure during spraying and the type of inert carrier (microcrystalline cellulose versus starch-based pellets) did not influence bacterial viability. A 5 % lactose matrix provided the best stabilisation of *L. lactis* after a 30 min layering process (remaining viability: 8.9 %), however if the process time was prolonged the addition of 10 % skim milk was needed to achieve a high survival rate. When increasing the concentration of bacteria in the layering suspension, a higher amount of stabilising matrix was required to maintain the viability of the microorganisms. If the formulation was stored for 12 months at -20°C and a relative humidity of 20 % the viability did not decrease, whereas storage at 8°C already resulted in a significant decrease of viability after 1 month storage. Microencapsulation of *L. lactis* Thy 12 (Chapter 2.3.3.) in a sodium alginate matrix yielded a similar viability compared to layering as processing technique, however the resulting particles were too sticky and not suitable for delivery of *L. lactis*.

3 As *L. lactis* Thy 12 is highly sensitive to the gastric environment, an enteric-coated formulation is required to deliver viable *L. lactis* to the intestine. Moreover, as Crohn's disease mainly affects the ileal part of the intestine, release must be targeted to this site. When the multi-particulate formulation was enteric coated with Eudragit® FS30D the viability decreased in function of coating thickness (Chapter 3.3.1.). Moreover, enteric protection was limited as only 1.1 % of the bacteria survived after 2 h in 0.1 N HCl. As the enteric coat was toxic for the bacteria as evidenced by the viability data, HPMC or PVA-based subcoats were applied to prevent direct contact between the bacteria and the enteric coat. Only Opadry® II did not significantly decrease the viability after subcoating, however after enteric coating of these subcoated pellets, the viability of the formulation was low.

To obtain ileum targeting (i.e. release at pH 6.8), two different approaches were used (Chapter 3.3.2.): decreasing the coating thickness and modifying the structure of the enteric polymer.

Although a coating thickness corresponding to 15 % polymer weight gain (Eudragit® FS30D) only resulted in complete release of the content of the pellets at pH 7.4, ileum targeting could be improved by reducing the coating thickness: drug release from pellets coated to a weight gain of 5 % resulted in complete release after 100 min in pH 7.0 phosphate buffer. Chemical modification of the Eudragit® FS30D polymer structure to increase the concentration of free carboxylic groups also resulted in a polymer suitable for ileum targeting.

4 In Chapter 4 a vaginal formulation for the delivery of probiotic *Lactobacillus* strains was developed. The behaviour of a multi-particulate pellet formulation (as novel vaginal delivery system for probiotics) was compared with a freeze-dried lactose/skim milk powder. An optimal vaginal formulation should distribute uniformly over the vaginal mucosa and be retained for a sufficient time to allow complete drug release in the vaginal cavity. Non-disintegrating microcrystalline cellulose pellets clustered around the vaginal fornix and 24 h after administration only a small fraction of the pellets could be detected in the vagina. In contrast, 24 h after administration, starch-based pellets had evenly distributed over the vaginal mucosa. After administration of HPMC or gelatine capsules filled with pellets vaginal disintegration of these systems was slow (in some cases over 6 h), probably related to the low amount of fluid present in the vagina. A clinical trial in healthy volunteers showed that starch-based pellets and freeze-dried lactose/skim milk powder did not disturb the vaginal pH and microflora.

5 Four different strains of *Lactobacillus* species (i.e. *L. jensenii*, *L. crispatus* 125, *L. crispatus* 128 and *L. gasseri*) were layered on inert carriers or freeze-dried using 10 % lactose/20 % skim milk as stabilising matrix. Immediately after drying, freeze-drying resulted in a higher survival compared to layering. Layering of *L. gasseri* was not

possible as the sticky nature of the pellets agglomerated the particles during processing. Long term storage of the *L. jensenii* and *L. crispatus* 128 strains was better for the layered pellets. After addition of vitamin C to the drying matrix, the *L. jensenii* layered formulation could be stored for 12 months at 4°C with only a limited drop in viability (0.5 log reduction). Centrifugation and tangential flow filtration were used to harvest the microorganisms from the growth medium and both resulted in similar process yields.

Layered starch-based pellets and freeze-dried powder, both containing 5×10^6 of *L. jensenii*, *L. crispatus* 125, *L. crispatus* 128, were vaginally administered to determine the colonisation potential of these formulations in healthy volunteers. The formulations were packed in capsules, administered on 5 consecutive days and the colonisation potential of the formulation was evaluated on day 6, 9, 12 and 33. Colonisation was only achieved with *L. jensenii*: in 7 of 16 volunteers this strain was detected in the vaginal microflora at all check-up days. As some of these volunteers (n=4) received the pellet and other the powder formulation (n=3) the formulation had no influence on vaginal colonisation. *L. crispatus* vaginally administered as a probiotic did not induce colonisation as a higher dose might be required to achieve this goal.

SAMENVATTING

1 Het doel van dit onderzoeksproject was de ontwikkeling van een multi-particulaire formulatie van melkzuurbacteriën: (a) de ontwikkeling van een orale formulatie met een hoge concentratie aan levende, genetisch gemodificeerde en IL-10 producerende *Lactococcus lactis* (b) de ontwikkeling van een vaginale formulatie van gezondheidsverbeterende melkzuurbacteriën (probiotica). Naast de bepaling van de leefbaarheid van de *Lactobacillus* stammen in de formulatie werd ook de vaginale retentie en distributie van de formulatie nagegaan, aangezien dit een essentiële parameter is voor een goede vaginale geneesmiddelvorm. Hoofdstuk 1 bespreekt het gebruik van genetische gemodificeerde *Lactococcus lactis* en probiotische *Lactobacillus* stammen als therapeutica in de behandeling van de ziekte van Crohn en bacteriële vaginose, respectievelijk. De strategieën om melkzuurbacteriën te stabiliseren in droge vorm en de productietechnieken gebruikt om de orale en vaginale formulatie te produceren worden besproken in dit inleidende hoofdstuk.

2 In Hoofdstuk 2 werd een multi-particulaire formulatie van levende *Lactococcus lactis* kiemen ontwikkeld via wervelbed layering op inerte dragers. Bacteriën werden gesuspendeerd in een stabiliserende matrix en vervolgens gesproeid en gedroogd op pellets. Deze multi-particulaire formulatie heeft als voordeel dat ze snel geklaard wordt uit de maag en bovendien kan deze formulatie in hetzelfde toestel maagsapresistent omhuld worden. Hoofdstuk 2.3.1. beschrijft de optimalisatie van de Bioscreen turbiditeitsmethode, die gebruikt wordt om de leefbaarheid van de gedroogde *L. lactis* kiemen te bepalen. In Hoofdstuk 2.3.2. werd het layeringsproces geoptimaliseerd door verschillende proces- en formulatieparameters te wijzigen. De atomiserende druk tijdens het sproeien en het type inerte drager (microkristallijne cellulose of zetmeelpellets) had geen invloed op de

leefbaarheid van de kiemen. Een 5 % lactose matrix resulteerde in de hoogste leefbaarheid van *L. lactis* na een layeringsproces van 30 min (leefbaarheid van 8,9 %), maar bij het verlengen van de procestijd tot 2 u was toevoeging van 10 % magere melk nodig om een hoge overleving te behouden. Wanneer de concentratie aan bacteriën in de layeringssuspensie verhoogd werd, was het tevens noodzakelijk om de hoeveelheid stabiliserende matrix te verhogen. Na bewaren van de formulatie voor 12 maand bij -20°C en een relatieve vochtigheid van 20 % was er geen daling in leefbaarheid, maar bewaring bij 8°C resulteerde reeds in een significante daling in leefbaarheid na 1 maand bewaring. De leefbaarheid na microencapsulatie van *L. lactis* (Hoofdstuk 2.3.3.) in een natriumalginaat matrix was vergelijkbaar met de leefbaarheid na layeren, maar de verkregen partikels waren te plakkerig en als dusdanig niet geschikt als bacteriële formulatie.

3 Aangezien de leefbaarheid van *L. lactis* aangetast wordt door de zure pH van de maag, moet de *L. lactis* formulatie omhuld worden met een maagsapresistent polymeer. Bovendien moet de vrijstelling van *L. lactis* getarget worden naar het ileum, aangezien deze zone van de dunne darm het meeste aangetast is bij de ziekte van Crohn. Omhullen van de gelayerde formulatie met Eudragit® FS30D, resulteerde in een daling in leefbaarheid bij toenemende omhullingdikte (Hoofdstuk 3.3.1.). Bovendien was de bescherming na 2 u 0.1 N HCl zwak, aangezien slechts 1,1 % van de kiemen overleefde. Aangezien de leefbaarheidresultaten wezen op een toxiciteit van het maagsapresistent polymeer, werd een HPMC of PVA subcoat aangebracht om het direct contact tussen de kiemen en de maagsapresistente coat te vermijden. Alleen Opadry® II resulteerde in geen significante daling na subcoaten, maar na omhullen met Eudragit® FS30D van deze gesubcoate pellets, werd slechts een lage leefbaarheid bekomen.

Om ileum targeting (i.e. vrijstelling bij pH 6.8) te bereiken werden 2 verschillende benaderingsmethoden gebruikt (Hoofdstuk 3.3.2.): verminderen van de dikte van de coat en modificatie van de structuur van het maagsapresistent polymeer. Een coatingdikte verkregen na omhullen tot een gewichtstoename van 15 % (Eudragit® FS30D) resulteerde pas in een volledige vrijstelling bij pH 7.4, maar na vermindering van de coating dikte werd ileum targeting verbeterd: na 100 min bij pH 7 was de vrijstelling van pellets omhuld tot een gewichtstoename van 5 % compleet. Stijging van het aantal vrije carboxylgroepen door middel van chemische modificatie van het Eudragit® FS30D polymeer, resulteerde in een polymeer geschikt voor ileum targeting.

4 In Hoofdstuk 4 werd een vaginale formulatie voor probiotische *Lactobacillus* stammen ontwikkeld. Het gedrag van een multi-particulaire pellet formulatie (als nieuw vaginaal toedieningsysteem voor probiotica) werd vergeleken met een gevriesdroogd lactose/magere melk poeder. Een optimale vaginale formulatie moet zich uniform spreiden over de vaginale mucosa en voldoende lang weerhouden worden om geneesmiddelvrijstelling te maximaliseren. Niet-desintegrerende microkristallijne cellulose pellets clusterden samen rond de vaginale fornix en 24 u na toediening werden slechts een kleine hoeveelheid pellets teruggevonden. In tegenstelling tot de niet-desintegrerende pellets, waren de zetmeelpellets uniform verspreid over de vaginale mucosa 24 u na toediening. Desintegratie van de gebruikte gelatine en HPMC capsules was traag (in sommige gevallen meer dan 6 u), vermoedelijk door de lage hoeveelheid vocht aanwezig in de vagina. In een klinische studie bij gezonde vrijwilligers werd aangetoond dat de zetmeelpellets en het gevriesdroogde lactose/ magere melk poeder de vaginale pH en microflora niet verstoren.

54 verschillende *Lactobacillus* stammen (i.e. *L. jensenii*, *L. crispatus* 125, *L. crispatus* 128 en *L. gasseri*) werden gelayerd op inerte dragers of gevriesdroogd waarbij 10 % lactose/20 % magere melk gebruikt werd als stabiliserende matrix. Direct na drogen resulteerde vriesdrogen in een hogere overleving dan layeren. Layeren van *L. gasseri* was onmogelijk omwille van het kleven van de pellets tijdens het layeringsproces. Na bewaring voor 9 maand bij 4°C was de overleving van *L. jensenii* en *L. crispatus* 128 beter voor de gelayerde pellets dan voor het gelyofiliseerde poeder. Na toevoeging van vitamine C aan de stabiliserende matrix kon de gelayerde formulatie van *L. jensenii* voor 12 maand bewaard worden bij 4°C, waarbij slechts een kleine daling in leefbaarheid werd waargenomen (0,5 log reductie). Centrifugeren en tangentiële filtratie werden gebruikt om de kiemen te collecteren uit het groeimedium, waarbij beide technieken resulteerden in een vergelijkbare opbrengst.

Gelayerde zetmeelpellets en gevriesdroogd poeder, beide beladen met 5×10^6 *L. jensenii*, *L. crispatus* 125 en *L. crispatus* 128, werden vaginaal toegediend om het koloniserend vermogen van beide formulaties te bepalen bij gezonde vrijwilligers. De formulaties werden afgevuld in capsules en gedurende 5 opeenvolgende dagen toegediend, waarbij het koloniserend vermogen bepaald werd op dag 6, 9, 12 en 33. Enkel voor *L. jensenii* werd kolonisatie bereikt: in 7 van de 16 vrijwilligers werd op elke onderzoeksdag deze stam teruggevonden in de vaginale flora. Aangezien sommige van deze vrijwilligers (n = 4) de pellet formulatie gebruikten en sommige de poeder formulatie (n = 3), werd besloten dat de formulatie zelf geen invloed heeft op de vaginale kolonisatie. Een hogere dosis aan *L. crispatus* zal waarschijnlijk nodig zijn om kolonisatie met deze stam te bereiken.