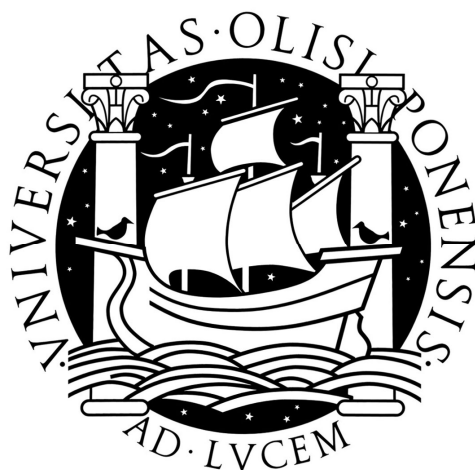


**UNIVERSIDADE DE LISBOA**

**FACULDADE DE FARMÁCIA**



**Evaluation of the viability of *Lactobacillus* spp. in  
different dosage forms**

**Mariya Ivanova Brachkova**

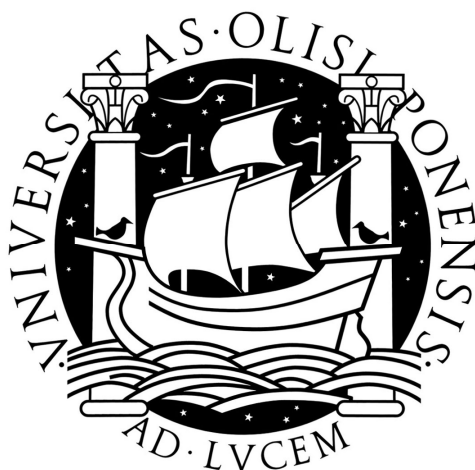
**Doctoral Thesis in Pharmacy  
(Pharmaceutical Technology)**

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**Evaluation of the viability of *Lactobacillus* spp. in  
different dosage forms**

**Mariya Ivanova Brachkova**

**Supervisor: Prof. Doctor João Pinto  
Co-supervisor: Prof. Doctor Aida Duarte**

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## Abstract

The Thesis aimed to provide evidence on the incorporation of *Lactobacillus* spp. into different dosage forms keeping their viability and antibacterial activity. In order to achieve this aim, the survival of probiotic microorganisms in freeze-dried powders, tablets, pellets, calcium alginate beads and films has been investigated. Two strategies were used: a) direct incorporation of bacterial suspensions into pellets, sodium alginate beads and films and b) incorporation of freeze-dried bacteria into tableting excipients to produce tablets.

The vegetative forms of *Lactobacillus plantarum*, *Lactobacillus rhamnosus* strain GG, *Lactobacillus lactis* and *Lactobacillus bulgaricus* and a spore form of *Bacillus subtilis* were considered as model bacterial strains. The different bacteria chosen have shown different viabilities to formulation and processing with decreasing viability as follows: *B. subtilis*, *L. plantarum*, *L. rhamnosus* GG and *L. bulgaricus*.

The process of drying was critical for obtaining a high survival rate. Freeze drying allowed high viability ( $\leq 1$  log unit decrease) contrary to air drying observed during the process of extrusion-spheronisation and subsequent fluid bed drying. Nutrients and cryoprotectants (skim milk, glycerol, mannose, calcium alginate) should be present at early stages of processing, before de-hydration, to promote the stabilization of bacteria. Pressure, whether applied during extrusion or compaction, reduced bacterial viable counts. Tableting of freeze-dried bacteria alone or together with inulin had a less deleterious effect on bacterial viability (1-2 log unit decrease during compaction followed by 1-2 log unit during storage) compared to pelletisation (in some cases 3 log units during processing, reaching minimum of detection in 1 to 4 months). However, tableting of freeze-dried bacteria together with microcrystalline cellulose had a detrimental effect on bacterial viability comparable to pelletisation. Coating of either tablets or pellets was considered less deleterious compared to tableting and pelletisation. Coating caused an initial decrease of viability ( $< 1$  log unit) but did not affect the survival rates further during storage. The highest survival rates of probiotic bacteria were obtained by the technique of immobilization into calcium alginate beads and films:  $\leq 1$  log unit decrease during freeze-drying of beads and films and  $< 1$  log unit during storage.

The antibacterial activity of lactobacilli against multi-resistant clinical isolates (upon their preservation in calcium alginate beads and films) has been observed. Lactobacilli, particularly whole cultures, at high cell concentrations ( $10^8$  cfu/ml) were inhibitory active. The antibacterial activity of the lactobacilli remained stable during processing and storage provided the viability was preserved (*L. plantarum*, LGG and *L. lactis*). Immobilized lactobacilli caused a 5 to 6 log unit reduction of a VIM-2-metallo- $\beta$ -lactamase producing *Pseudomonas aeruginosa* in rat burns: from  $10^7$ - $10^8$  to  $10^2$  *P. aeruginosa* / g of skin in 8 hours.

It has been demonstrated the successful production of various dosage forms for the delivery of viable lactobacilli with antibacterial activity against multi-resistant bacteria. The developed dosage forms could contribute to the use of lactobacilli as an alternative in topical prevention of burn wound infections.

**Keywords:** antibacterial activity, burn wound, calcium alginate immobilisation, *Lactobacillus* spp., mini-tablets, multi-resistant clinical isolates, pellets, viability.

## Resumo

O objectivo desta tese é demonstrar a incorporação de *Lactobacillus* spp. em diferentes formas farmacêuticas mantendo a sua viabilidade e actividade antibacteriana. Para obter este objectivo, foi investigada a sobrevivência dos microrganismos probióticos em pós liofilizados, comprimidos, pellets, beads e filmes de alginato de cálcio. Foram usadas duas estratégias: a) incorporação directa de suspensões bacterianas para produzir pellets, beads e filmes de alginato de cálcio e b) incorporação de bactérias liofilizadas em excipientes para produzir comprimidos.

As formas vegetativas *Lactobacillus plantarum*, *Lactobacillus rhamnosus* estirpe GG, *Lactobacillus lactis* e *Lactobacillus bulgaricus* e *Bacillus subtilis* em forma de esporos foram considerados como modelo de estirpe bacteriana. As diferentes bactérias seleccionadas demonstraram diferentes viabilidades à formulação e produção, com viabilidade decrescente na seguinte sequência: *B. subtilis*, *L. plantarum*, *L. rhamnosus* GG e *L. bulgaricus*.

O processo de secagem foi crítico para a obtenção de altas taxas de sobrevivência. Liofilização permitiu alta viabilidade ( $\leq 1$  unidade logarítmica de decréscimo) contrariamente à secagem ao ar observada durante o processo de extrusão e esferonização e subsequente secagem por leito fluido. Nutrientes e crioprotectores (skim milk, glicerol, manose, alginato de cálcio) devem estar presentes nas fases iniciais do processo, antes da desidratação, para promover a estabilização das bactérias. A pressão, aplicada durante a extrusão ou compactação, reduziu a viabilidade. A produção de comprimidos de bactérias liofilizadas com ou sem inulina teve um menor efeito deletério na viabilidade (1 a 2 unidades logarítmicas de decréscimo durante a compactação seguida por 1 a 2 unidades logarítmicas de decréscimo durante armazenamento) quando comparada com a peletização (3 unidades logarítmicas de decréscimo nalguns casos, atingindo a detecção mínima ao fim de 1 a 4 meses). No entanto, a produção de comprimidos de bactérias liofilizadas em conjunto com celulose microcristalina teve um efeito deletério na viabilidade comparável ao da peletização. O revestimento de comprimidos ou pellets foi considerado menos deletério do que a produção de comprimidos ou peletização. O revestimento causou um decréscimo inicial de viabilidade ( $< 1$  unidade logarítmica) mas não afectou as taxas de sobrevivência durante o armazenamento. As mais altas taxas de sobrevivência de bactérias probióticas foram obtidas com a técnica de imobilização em beads e filmes de alginato de cálcio:  $\leq 1$

unidade logarítmica de decréscimo durante liofilização de beads e filmes e <1 unidade logarítmica durante armazenamento.

Foi observada a actividade antibacteriana de lactobacilli contra isolados clínicos multi-resistentes (após a sua preservação em beads e filmes de alginato de cálcio). Observou-se que lactobacilli, particularmente as culturas inteiras, em altas concentrações celulares ( $10^8$  cfu/ml) provocaram inibição. A actividade antibacteriana dos lactobacilli permaneceu estável durante o processamento e armazenamento desde que a viabilidade fosse preservada (*L. plantarum*, LGG e *L. lactis*). Lactobacilli imobilizados causaram um decréscimo de 5 a 6 unidades logarítmicas de VIM-2-metallo- $\beta$ -lactamase produzindo *Pseudomonas aeruginosa* em queimaduras de ratos: de  $10^7$ - $10^8$  a  $10^2$  *P. aeruginosa* / g de pele em 8 horas.

Foi demonstrada a produção com sucesso de várias formas farmacêuticas para a administração de lactobacilli viáveis com actividade inibitória contra bactérias multi-resistentes. As formas de dosagem desenvolvidas podem contribuir para o uso tópico de lactobacilli como alternativa à prevenção de infecções em queimaduras.

**Palavras chave:** actividade antibacteriana, imobilização em alginato de cálcio, isolados clínicos multi-resistentes, *Lactobacillus* spp., mini-comprimidos, pellets, queimadura, viabilidade.



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## List of Abbreviations

AAD:	Antibiotic-associated diarrhea
ALT:	Alanine aminotrasferase
AST:	Aspartate aminotransferase
ATCC:	American type culture collection
AU:	Arbitrary units
BHI:	Brain heart infusion
CFU:	Colony forming units
CIP:	Collection de l'Institut Pasteur
cm:	Centimetre
DCs:	Dendritic cells
dl:	Decilitre
DMH	1,2-dimethylhydrazine
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
FD:	Freeze-drying
FFUL:	Faculty of Pharmacy, University of Lisbon
g:	Gram
G+C:	Guanine plus cytosine
h:	Hour
HIV-1:	Human immune deficiency virus, type 1
IBD:	Inflammatory bowel disease
IC:	Immobilised cells
Ig:	Immunoglobulin
IL:	Interleukin
INF- $\gamma$ :	Interferon $\gamma$
Inulin HP:	High performance inulin
Inulin ST:	Standard chicory form of inulin
IU:	International unit
J:	Joule
kg:	Kilogram
l:	Litre
LAB:	Lactic acid bacteria
LPMC:	Lamina propria mononuclear cells

m:	Metre
M:	Molar (1 M = 1 mole/litre)
mA:	Milliamper
mbar:	Millibar
MCC:	Microcrystalline cellulose
MDCs:	Myeloid dendritic cells
mg:	Milligram
MH:	Mueller-Hinton
min:	Minute
ml:	Millilitre
mm:	Millimetre
MNNG:	N-methyl-N-nitro-N-nitrosoguanidine
MRS:	de Man-Rogosa-Sharpe
N:	Newton
osc:	Oscillation
Pa:	Pascal
PBMC:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
PP:	Payer's patches
RNA:	Ribonucleic acid
s:	Second
SD:	Standard deviation
SE:	Standard error
SEM:	Scanning electron microscopy
SM:	Skim milk
SPP:	Species
SUBSP:	Subspecies
TLR:	Toll-like receptor
TTFC:	Tetanus toxin fragment C
UC:	Ulcerative colitis
µl:	Microlitre

## **CHAPTER 1**

### **LACTOBACILLI AND OTHER PROBIOTIC BACTERIA**

## 1.1 General considerations of lactobacilli and other probiotic bacteria

A beneficial association of microorganisms with the human host was suggested by Metchnikoff in 1907. He considered the longevity of the Bulgarian people to be related to the high intake of fermented milk products containing *Bacillus bulgaricus*. About 100 years later there has been a growing interest in beneficial bacteria and their effects. The term probiotic has been introduced and defined as live microorganisms that, when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). In order to qualify as probiotic, microorganisms should fulfil most, if not all, of the criteria listed in Table 1.1. The established probiotics that meet these criteria are generally lactic acid bacteria (LAB), most commonly *Lactobacillus* and *Bifidobacterium* species, but *Lactococcus*, *Streptococcus* and *Enterococcus* species, as well as some non-pathogenic strains of *Escherichia coli* and certain yeast strains also qualify. There has been increasing evidence that probiotic strains can exhibit the same activities as commensal bacteria, including immunomodulation and protection of the host from pathogens by competitive exclusion (Borchers et al., 2009).

Table 1.1 Criteria for the use of probiotics in humans.

---

Identified at the genus, species and strain level
Strain should be deposited in an international culture collection;
Safe for food and clinical use
Non pathogenic; Non degrading the intestinal mucosa; Not carrying transferable antibiotic resistance genes; Not conjugating bile acids; Susceptible to antibiotics
Able to survive intestinal transit
Acid and bile tolerant
Able to adhere to mucosal surfaces
Able to colonize the human intestine or vagina (at least temporarily)
Producing antimicrobial substances
Able to antagonize pathogenic bacteria
Possessing clinically documented and validated health effects
Stable during processing

---

(adapted after Borchers et al., 2009)

The present work will be focused on *Lactobacillus* species as main representatives of probiotic bacteria. Their application as bacteriotherapy and methods for stabilization into delivery systems will be reviewed in this chapter.

### 1.1.1 Genus *Lactobacillus*

The genus *Lactobacillus* is the largest group among the *Lactobacillaceae*, and contains over 100 species (Felis and Dellaglio, 2007). Lactobacilli are Gram-positive, microaerophilic, non-spore forming rod-shaped bacteria, for which motility is uncommon. However, when present, motility is performed by peritrichous flagella. Cells, varying from long and slender, sometimes bent to short, often coryneform coccobacilli, often form chains (Figure 1.1).

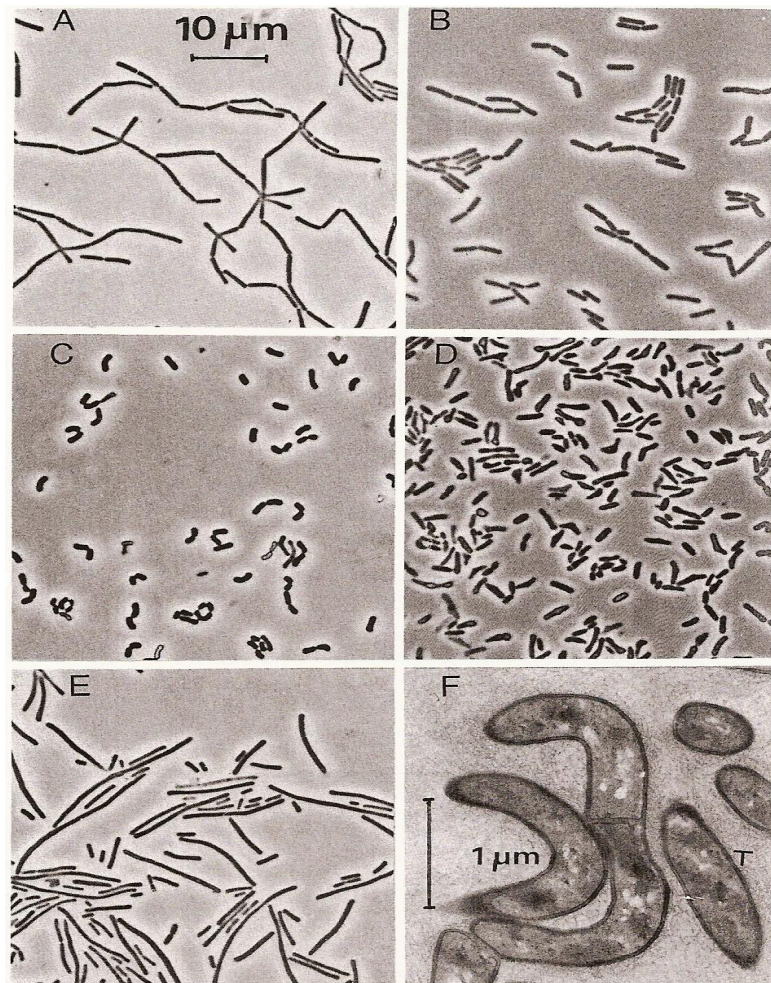


Figure 1.1 Phase contrast (A-E) and electronic micrographs (F) showing different cell morphology of lactobacilli (A, *L. gasseri*; B, *L. agilis*; C, *L. curvatus*; D, *L. minor*; E, *L. fermentum*; F, involution form of lactobacilli in a thin section of kefir grain) (after Kandler and Weiss, 1986).

Growth temperature ranges from 2 to 53°C with optimum generally between 30 and 40°C. Optimal pH usually ranges from 5.5 to 6.2. Growth generally occurs at 5.0 or less and is often reduced at neutral or initially alkaline reactions. Lactobacilli are found in dairy products, grain products, meat and fish products, water, sewage, beer, wine, fruits and fruit juices, pickled vegetables, sauerkraut, silage, sour dough, and mash. Species



### 1.1.2 Bacteriotherapy

The competition among microorganisms and interaction with mammalian host has been evolving for millions of years. The fact that we have survived is in no small part owing to the ability of commensal bacteria to protect the host from microbial induced diseases (Reid et al., 2001). Starting from the first hours after the delivery the interaction of the host with microorganisms begins. This interaction leads to colonisation of host epithelial surfaces. Of these surfaces mucosa represents in humans about 300 m<sup>2</sup> while skin covers approximately 2 m<sup>2</sup> surface of the human body. Human individuals are consequently complex ecosystems formed by 10<sup>13</sup> mammalian cells and by commensal microflora (approximately 10<sup>14</sup> microbes) (Tlaskalová-Hogenová et al., 2004). Commensal microflora is an integral part of a complex of natural mechanisms on mucosal surfaces and skin that at an optimal composition prevents attachment and colonisation of pathogenic microorganisms and their invasion into epithelial cells and circulation. Some of these pathogens are already part of the indigenous microflora (staphylococci, streptococci, *Corynebacterium*; Gram-negative cocci), whereas others, such as *Salmonella*, are truly foreign invaders. Beside the direct interaction with pathogenic bacteria commensal microflora plays an important role in anti-infectious resistance by its influence on immune system. Commensal microflora stimulates the development of both local and systemic immunity and later on, it evokes, on the contrary, regulatory (inhibitory) mechanisms (oral, mucosal tolerance) intended to keep both mucosal and systemic immunity in balance (Tlaskalová-Hogenová et al., 2004). The significant role of the host microflora for resistance to disease has been well illustrated by Collins and Carter in 1978. The introduction of less than 10 viable *Salmonella enteridis* into the germfree mice intestinal tract represented a 100% lethal dose, whereas 1 x 10<sup>9</sup> bacteria were required to kill animals with complete intestinal microflora.

The idea to use harmless commensal or probiotic bacteria to replace pathogenic bacteria at skin and mucosa has emerged from microbial ecology and competitive exclusion studies. Bacterial interference is an increasingly attractive approach for prevention and therapy (Reid et al., 2001; Huovinen, 2001; Peral et al., 2009; Huang et al., 2009; Skovbjerg et al., 2009). In this respect lactobacilli, as part of the commensal microbial flora of the intestinal tract of humans and mammals and main representatives of probiotic bacteria, can be useful candidates (Peral et al., 2009; Huang et al., 2009).

Lactobacilli are considered as GRAS (Generally Recognized As Safe) organisms with a very long record of safe oral consumption. They are shown to modulate host immunity and neutralize pathogens. Furthermore, lactobacilli confer the advantage that they can be engineered to deliver therapeutics.

### **1.1.2.1 Probiotic bacteria and immunity**

Numerous studies have demonstrated that probiotic bacteria enhance non-specific and specific host immune responses to microbial pathogens. The oral pre-treatment with a lysozyme lysate from *L. bulgaricus* (Deodan) activated the phagocytic and secretory functions of mononuclear cells and decreased the mortality in mice infected intraperitoneally with *Klebsiella pneumoniae* and *Listeria monocytogenes* (30% mortality of Deodan treated mice vs 70% in control group infected with *K. pneumoniae* and 80% with *L. monocytogenes*) (Popova et al., 1993). The consumption of *L. acidophilus* (Schiffrin et al., 1995) and *L. johnsonii* (Donnet-Hughes et al., 1999) increased leukocyte phagocytic activity in healthy adults. Phagocytosis is responsible for early activation of the inflammatory response before antibody production. Phagocytes release toxic agents, e.g. reactive oxygen intermediates and lytic enzymes, in various inflammatory reactions. Phagocytic activity results in further recruitment of immunocompetent cells and the generation of inflammatory response. Gill et al., 2000, observed that supplementation with *L. rhamnosus*, *L. acidophilus* and *Bifidobacterium lactis* resulted in increase in phagocytic activity of peripheral blood leucocytes and peritoneal macrophages, NK (natural killer)-cell activity, IFN- $\gamma$  (interferon- $\gamma$ ) production and antibody responses to orally (cholera toxin) and systemically (tetanus vaccine) administered antigens in healthy mice.

It has been shown that orally ingested probiotic bacteria can activate immune responses at distant mucosal sites. This phenomenon is based on one impressive feature of the immune system, which is the mobility of its cells. Following antigen stimulation in Payer's patches (PP) and its presentation to B and T cells, the antigen induced B and T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) are able to migrate via efferent lymphatics and through the mesenteric node; they reach the systemic circulation through the thoracic duct and repopulate not only the lamina propria of the intestine but other distant mucosal sites such as respiratory, urogenital, exocrine glands such as lachrymal, salivary, mammary and prostatic glands (Brandtzaeg and Pabst, 2004). This phenomenon has been called



the Common Mucosal System. It can explain the observed by Gluck and Gebbers, 2003, reduction (19%) in the occurrence of nasal potentially pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*,  $\beta$ -haemolytic streptococci and *Haemophilus influenzae*) after ingestion of a probiotic drink containing *Lactobacillus rhamnosus* strain GG (LGG), *Streptococcus thermophilus*, *L. acidophilus* and *Bifidobacterium*.

Epithelial intestinal cells are another important component of the mucosal immune system. The epithelial cells communicate with other mucosal cells via a spectrum of mediators that act on the intestinal epithelial cells as well as on the intraepithelial lymphocytes, lymphoid cells, and mononuclear phagocytes. The cascade of mediators is regulated to induce or to down-regulate appropriate host immune and inflammatory responses at mucosal surfaces (Perdigon et al., 2001). Whatever the route of the secretory immune response induction, the result is that by oral stimulation, distant mucosal sites can be repopulated with IgA producing cells to protect these surfaces. Oral administration of *L. casei*, *L. bulgaricus*, *L. plantarum*, *L. rhamnosus*, *Lactococcus lactis* and *S. thermophilus* induced an increase in the sIgA cells in the bronchus of mice. The increase of the CD4<sup>+</sup> cells induced by *L. plantarum*, *L. rhamnosus* and *S. thermophilus* suggested interaction at the Payer's patches. No specific antibodies were determined against *L. lactis* and *L. bulgaricus*. Thus, these bacteria have been bound to epithelial cells that activated B lymphocytes without processing and presenting their epitops (Perdigon et al., 1999).

In spite of the ability of the common mucosal system to induce a good local mucosal response, local stimulation is also required. In 2005 de Moreno de LeBlanc et al. demonstrated immunoregulatory capacity of oral administered milk fermented by *L. helveticus* on the immune response in mammary glands in presence of a local pathology (breast tumour). Mice fed with *L. helveticus* fermented milk and injected with breast tumour cells increased IgA and CD4 positive cells, tumour control increased CD8<sup>+</sup> cells, while mice from fermented milk control group (without tumour cell injection) did not show changes in immune cell or cytokine positive cell numbers. IL (interleukin)-10 increase and IL-6 decrease were more pronounced in mice fed with fermented milk than in the other groups.

The release of cytokines by the immune cells plays an important role in triggering the immune response. Cytokine interventions could be used to stimulate or suppress immunity. Probiotic bacteria have been reported to modulate the immunological

functions of dendritic cells (DCs), which are critical players in both innate and adaptive immunity since they are the most potent antigen-presenting cells. Co-incubation experiments result in enhancement or inhibition of dendritic cell maturation and IL-12 or IL-10 induction (Borchers et al., 2009). IL-12 is a proinflammatory cytokine that can induce a  $T_H1$  response.  $T_H1$  cells, also known as Tdth (delayed type hypersensitivity), are involved in cell mediated immune responses such as destruction of intracellular bacteria, some types of delayed hypersensitivities, autoimmune diseases and the rejection of organ transplants. Mohamadzadeh et al., 2005 investigated the effect of *L. gasei*, *L. johnsonii* and *L. reuteri* on human myeloid dendritic cells (MDCs). Lactobacilli exposed MDCs secreted high levels of IL-12 and IL-18 but not IL-10. MDCs activated with lactobacilli clearly skewed  $CD4^+$  and  $CD8^+$  T cells to  $T_H1$  and Tc1 (cytotoxic) polarization. Such strains promoting DCs to regulate T cell responses to cytotoxic pathways could be particularly advantageous as vaccine adjuvants, in infectious diseases and malignancy.

In contrast, in other situations such as autoimmune disease or inflammatory disorders, the inhibition of certain cytokines might be desirable. For example, IL-10 exhibits an impressive ability to inhibit the production by  $CD4$  T cells ( $T_H1$  and  $T_H2$  (T helper cells)) and macrophages of both inflammatory cytokines (IL-1, IL-8 and TNF- $\alpha$  (tumor necrosis factor-  $\alpha$ )) and those inducing cell mediating cytotoxicity (IL-2 and IFN- $\gamma$ ). Moreover, IL-10 production by DCs can induce regulatory T cells important for induction of tolerance. Hoarau et al., 2006 found that the supernatant from the culture of *Bifidobacterium breve* C50 could induce human dendritic cell (DC) maturation with high IL-10 and low IL-12 production. Analogically to *B. breve*, *L. rhamnosus* resulted in low IL-12 and IL-18 production by mature DCs, which could explain anti-inflammatory effect of the strain in gastrointestinal disease and allergy (Braat et al., 2004).

### **1.1.2.2 Antimicrobial effect of probiotic bacteria**

The ability of lactobacilli to produce antimicrobial substances has historically long been used to preserve foods. Fermentation reduces the amount of available carbohydrates and results in a range of small molecular weight organic molecules that exhibit antimicrobial activity. The biological significance is believed to be of

amensalism, a means of one bacterium gaining advantage over another competing microbe (Ouweland and Vesterlund, 2004).

Homofermentative lactobacilli ferment hexoses exclusively to lactic acid, while heterofermentative to equimolar amounts of lactic acid, acetic acid / ethanol and CO<sub>2</sub> (Kandler and Weiss, 1986). Acids have been shown to diffuse passively through the cell membrane. After entry into the cytoplasm they dissociate into protons and charged derivatives to which the cell membrane is impermeable. The intracellular accumulation of protons leads to internal acidification which reduces the activity of acid-sensitive enzymes and damages proteins and DNA. The accumulation in the cytoplasm of the anionic moiety of the dissociated organic acids was suggested to affect the cellular physiology through a chelating interaction with essential elements (de Guchte et al., 2002). Limit pH values under which bacteria do not grow are species dependent: *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Brevibacterium lineus* and *Micrococcus* spp (5.5-5.6); *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus vulgaris* (4.4); *Salmonella typhi* (4.0-4.5); *Salmonella paratyphi*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Clostridium botulinum*, *Enterococcus* spp. (4.5-4.9); *Lactobacillus* spp. (3.8-4.4); *Staphylococcus aureus* (4.0); *Lactococcus lactis* (4.3-4.8) (Nemcova, 1997). In addition to its antimicrobial property due to the lowering of the pH, lactic acid functions also as a permeabilizer of the Gram-negative bacterial outer membrane (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhimurium) (Alakomi et al., 2000). The permeabilizing capacity of lactic acid can promote the antimicrobial activity of other fermentative metabolites that are too lipophilic or too large to effectively penetrate the intact Gram-negative bacterial outer membrane but that could possibly do so in the presence of lactic acid. Makras et al., 2006, observed that the antibacterial activity of *L. acidophilus*, *L. amylovorus*, *L. casei* Shirota and *L. rhamnosus* GG against *Salmonella enterica* serovar Typhimurium was solely due to the production of lactic acid. However, the antibacterial activity of *L. johnsonii* and *L. plantarum* was due to the production of lactic acid and a non-proteinaceous, heat-stable inhibitory substance, which was only active in the presence of lactic acid.

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is one of the toxic derivatives of oxygen generated by lactic acid bacteria growing aerobically (Otero and Nader-Macias, 2006). The bactericidal effect of hydrogen peroxide is owing to its strong oxidizing effect on the bacterial cell: sulfhydryl groups of cell proteins and membrane can be oxidized

(Ouwehand and Vesterlund, 2004). Colonization of the urogenital tract by H<sub>2</sub>O<sub>2</sub> producing lactobacilli has been found to decrease the acquisition of human immune deficiency virus type 1 (HIV-1) infection (Martin et al., 1999), chlamydial and gonorrhoea infection (Wiesenfeld et al., 2003).

Lactobacilli produce a wide variety of antibacterial peptides. The peptide bacteriocins are inhibitory active at concentrations in the nanomolar range, and cause permeabilization and leakage of intracellular components in sensitive cells. They are commonly classified into three groups: class I, e.g. nisin, includes lantibiotics; class II, e.g. pediocin, small heat stable-peptides; and class III, e.g. helveticin J, large heat-labile proteins (Parente and Ricciardi, 1999). By definition, lactobacilli bacteriocins are extracellularly released primary or modified products of bacterial ribosomal synthesis and their inhibitory spectrum is in many cases limited to bacteria closely related to the producing strain (Nes and Holo, 2000). Only a few bacteriocins with wide inhibitory spectrum have been reported. *L. plantarum* strains isolated from the brine of spoiled black olives produced bacteriocins which inhibited the growth of Gram-positive (*Enterococcus faecalis*, *L. casei*, *Streptococcus pneumoniae*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) (Todorov and Dicks, 2005). *L. plantarum*, *L. pentosus*, *L. rhamnosus* and *L. paracasei* isolated from boza, a Balkan beverage, produced bacteriocins active against *L. casei*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Todorov and Dicks, 2006). *L. delbrueckii*, isolated from Bulgarian yellow cheese, produced bacteriocin active against *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Yersinia enterocolitica* and *Y. pseudotuberculosis* (Miteva et al., 1998). A bacteriocin called bulgarican produced by *L. bulgaricus* inhibited the growth of *Bacillus* spp, *Proteus* spp, *Salmonella* spp, *Staphylococcus* spp, *Streptococcus* spp, *Clostridium* spp, *Pseudomonas* spp, *Sarcina* spp, *Serratia* spp, *Lactobacillus* spp and *E. coli* (Nemcova, 1997).

Bacteriocin production is growth associated: it usually occurs throughout the growth phase and ceases at the end of the exponential phase or sometimes before the end of growth. A decrease of bacteriocin titre usually follows. This may be related to adsorption on producer cells or to degradation by proteases (Parente and Ricciardi, 1999). Bacteriocin production and activity are deeply affected by the type and level of the carbon, nitrogen and phosphate sources, cations and surfactants (De Vuyst, 1994; Parente and Ricciardi, 1999). Low bacteriocin activities (800AU/ml; 200AU/ml;

200AU/ml) were recorded when growing *L. plantarum* in BHI (Brain Heart infusion), M17 (medium for lactic streptococci) and 10% soy milk, respectively. The strain produced the highest activity of its bacteriocin (25 600 AU/ml) in MRS broth (Todorov and Dicks, 2005). Furthermore, growth at optimal temperature usually results in optimal bacteriocin production (Todorov and Dicks, 2005). Some lactic acid bacteria produce more than one bacteriocin. *L. plantarum* produced high levels of plantaricin S during log phase and also produced another bacteriocin (plantaricin T) with a different activity spectrum in late stationary phase (Jiménez-Díaz et al., 1993). Another strain of *L. plantarum* was found to produce two two-peptide bacteriocins. The complementary peptides were most active when combined than when present individually (Anderssen et al., 1997). On the other hand a given bacteriocin can be produced by several strains or species. De Vuyst, 1994, screened 21 nisin producing and 6 non-producing strains of *Lactococcus lactis*. Nisin titres varied from 1 to 1886 IU/ml.

Another mechanism of lactobacilli antimicrobial activity involves the secretion of cell-cell signalling molecules produced in the course of interference with competing bacteria. In 2002 Gan et al. described inhibition of surgical *S. aureus* implant infection by secreted biosurfactant by *L. reuteri* (formerly *L. fermentum*). Four years later, Laughton et al., 2006, investigating the communication events between *L. reuteri* and *S. aureus*, found that lactobacilli could attenuate the pathogenic bacteria by changing the gene expression and inhibiting factors of virulence like superantigen-like proteins of *S. aureus* due to production of signal molecules.

### **1.1.2.3 Probiotic bacteria in allergic diseases**

Some gastrointestinal abnormalities have been observed in children with atopic eczema. These have included histological and functional abnormalities of the gastric epithelium, mild degrees of jejunal villous atrophy with eosinophil infiltration and oedema of the lamina propria, low jejunal mucosal IgA plasma cell counts in the duodenal mucosa, increased gastrointestinal permeation. Caffarelli et al., 1998, suggested that such gastrointestinal abnormalities could precede atopic eczema and even play a role in the aetiology of the skin disease by allowing allergic sensitisation as a result of increased antigen entry through the damaged mucosa. Kalliomaki et al., 2001a, suggested a crucial role of the balance of indigenous intestinal bacteria for the maturation of the human immunity to nonatopic mode. Reduced ratio of bifidobacteria

to clostridia at neonatal age preceded the later development of atopic sensitisation. Atopic subjects had more clostridia ( $3.8\text{-}22.9 \times 10^7$  vs  $1.8\text{-}6.1 \times 10^7$ ) and tended to have fewer bifidobacteria ( $0.4\text{-}7.6 \times 10^9$  vs  $2.5\text{-}14.6 \times 10^9$ ) in their stools than nonatopic subjects. These findings initiated investigation of the therapeutic effect of oral bacteriotherapy on atopic disease. Kalliomaki et al., 2001b, reported that *Lactobacillus* GG (LGG) was effective in prevention of early atopic disease in children at high risk. LGG was given prenatally to mothers who had at least one first-degree relative (or partner) with atopic eczema, allergic rhinitis or asthma, and postnatally for six months to their infants. The frequency of atopic eczema in the probiotic group was half that of the placebo group. In 2003 Rosenfeldt et al., observed that *L. rhamnosus* and *L. reuteri* at a dose of  $10^{10}$  cfu of each strain given in combination for 6 weeks to 1- to 13-year-old children with atopic dermatitis resulted in experienced improvement of the eczema in 56% of the patients. In 1997 Majamaa and Isolauri demonstrated the favourable effect of LGG administered to infants with atopic eczema and cow's milk allergy. LGG was added in their extensively hydrolysed whey formula in a concentration of  $5 \times 10^8$  cfu/g formula. The clinical score of atopic dermatitis improved significantly during the 1-month of treatment. The median concentration of faecal tumour necrosis factor-alpha decreased significantly in this group, from 709 pg/g (91 to 1131 pg/g) to 34 pg/g (19 to 103 pg/g), but not in those receiving the extensively hydrolysed whey formula only.

Treatment of pregnant mothers and their high-risk infants with a combination of probiotics (LGG, *L. rhamnosus*, *B. breve*, and *Propionibacterium freudenreichii* subsp. *Shermanii*) and prebiotic (galacto-oligosaccharides) showed no overall preventive effect on allergic diseases by age of 2 years but tended to reduce IgE-associated atopic diseases. The increase of the proportion of asymptomatic infants showed that probiotics seemed not to affect sensitization per se but regulated the path from sensitization to clinical disease (Kukkonen et al., 2007).

In contrast to the discussed findings, Kopp et al., 2008, have shown that probiotic LGG had no preventive effect on the development or severity of atopic disease in infants (2 years old) at high risk. Instead there was a significantly higher risk of  $\geq 5$  episodes with wheezing bronchitis in the LGG group, as compared with placebo.

#### 1.1.2.4 Probiotics in inflammatory bowel disease

The main characteristics of inflammatory and autoimmune diseases are tissue destruction and functional impairment as a consequence of immunologically mediated mechanisms which are principally the same as those functioning against pathogens. One of the main explanations for autoimmune phenomena is based on various infections capable of initiating the process in genetically predisposed individuals. Increased interest in infectious agents as causes of chronic diseases was awakened by the discovery of *Helicobacter pylori* as a causative agent of stomach ulcer, chronic gastritis and probably also of gastric cancer. Infection with intestinal pathogens such as *Salmonella*, *Shigella* and *Yersinia* can trigger autoimmune reactions in joints and other organs. Diseases with autoimmune features such as rheumatic fever and acute glomerulonephritis may develop after streptococcal infection (Tlaskalová-Hogenová et al., 2004). Dysregulation of intestinal immune response to normal bacterial flora was suggested to play a crucial role in inflammatory bowel disease (IBD). Duchmann et al., 1995, observed that lamina propria mononuclear cells (LPMC) isolated from inflamed intestine but not peripheral blood mononuclear cells (PBMC) of IBD patients with active inflammatory disease strongly proliferated after co-culture with sonicates of bacteria from autologous intestine. LPMC from adjacent non-inflamed intestinal areas of the same IBD patients and PBMC or LPMC isolated from non-inflamed intestine of controls and patients with IBD in remission, in contrast, did not proliferate. PBMC or LPMC which had been tolerant to bacteria from autologous intestine, however, strongly proliferated after co-culture with bacterial sonicates from heterologous intestine. These study shows that tolerance selectively exists to own but not to foreign intestinal flora and that tolerance is lost in intestinal inflammation.

Crohn's disease and ulcerative colitis are two distinct clinical forms of inflammatory bowel disease, characterised by chronic relapsing intestinal inflammation. Decreased levels of *Bifidobacterium* and *Lactobacillus* strains have been described, whereas raised counts of *Bacteroides* and *Enterococcus* species are found in inflamed mucosa of patients with IBD (Borchers et al., 2009). Because of potential of certain probiotic strains to influence the intestinal microbial balance, improve mucosal barrier function, and modulate immune responses, there have been numerous clinical trials of probiotic supplementation in IBD patients. Cui et al., 2004, have investigated the effect of bifidobacteria in patients with ulcerative colitis (UC). Thirty patients received treatment

with sulphasalazine and glucocorticoid and then were randomly administered bifidobacteria or placebo (starch) for eight weeks. Three patients (20%) in the probiotic group had relapses during the 2-month follow-up period, compared with 14 (93.3%) in the placebo group. The concentration of the faecal lactobacilli and bifidobacteria has been significantly increased in the probiotic treated group. The anti-inflammatory cytokines were elevated in comparison with the control group. Kruis et al., 2004, compared the efficacy in maintaining remission of the probiotic *Escherichia coli* strain Nissle and established therapy with mesalazine in patients with UC. 327 patients received either the probiotic drug or mesalazine. The study lasted for 12 months. Analysis revealed relapses in 40/110 (36.4%) patients in *E. coli* Nissle group and 38/112 (33.9%) in the mesalazine group. Consequently the efficacy of the probiotic *E. coli* Nissle in maintaining remission was equivalent to the gold standard mesalazine in patients with UC.

Gupta et al., 2000, demonstrated efficacy of LGG ( $10^{10}$  cfu during 6 months) in four children with mildly to moderately active Crohn's disease. There was a significant improvement 1 week after starting LGG, which was sustained through the study period. Median pediatric Crohn's disease activity index scores were 73% lower than baseline. Intestinal permeability improved in an almost parallel mode. Two years later Guandalini et al., 2002, confirmed the favourable effect of LGG in four children with active Crohn's disease.

In Crohn's disease, the potent proinflammatory cytokine (TNF- $\alpha$ ) seems to play a pivotal role in the pathogenesis of altered mucosal immune function. Anti- TNF- $\alpha$  monoclonal antibody therapy has shown a beneficial response associated with clinical, endoscopic and histological remission. Basing on that data, Borruel et al., 2002, obtained ileal specimens at surgery from patients with Crohn's disease. Mucosal explants from each specimen were cultured for 24h with non-pathogenic *E. coli*, *L. casei*, *L. bulgaricus* or *L. crispus*. The number of CD4 cells and release of TNF- $\alpha$  by inflamed Crohn's disease mucosa was significantly reduced by co-culture with *L. casei* or *L. bulgaricus*; changes induced by *L. crispus* or *E. coli* were not significant. None of the bacteria induced changes in non-inflamed mucosa.

Although the described studies were promising, the treatment of IBD with probiotic preparations proved to be difficult. The reports of Schultz et al., 2004, and Rolfe et al., 2006, suggested no benefit of LGG, *E. coli* Nissle, VSL#3, *Saccharomyces boulardii* on



induction or maintenance of remission to patients with Crohn's disease. Limited evidence that probiotics may reduce UC activity was reported by Mallon et al., 2007.

Findings are too variable to allow firm conclusions concerning the effectiveness of specific probiotics in IBD and allergic diseases. A possible explanation is that human responses to probiotic treatment might differ depending on the composition of the individuals' flora (Borchers et al., 2009).

#### **1.1.2.5 Probiotic bacteria in colon cancer**

The development of colon cancer is a multistage process that occurs when accumulation of mutation in certain protooncogenes and tumour suppressor genes leads to a cancer initiation (Wollowski et al., 2001). Probiotic bacteria have been investigated extensively in model systems for their ability to prevent mutations. In vitro assay systems using streptomycin-dependent strains of *Salmonella typhimurium* (TA98 and TA100) have shown antimutagenic activity of *L. bulgaricus*, *Streptococcus thermophilus*, *L. helveticus* and *L. acidophilus* against 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, 4-nitroquinoline-N-oxide, 2-aminofluorene, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), 3,2-dimethyl 1-4-amino-biphenyl and faecal mutagenic extracts from cats, monkeys and dogs (Hosono and Kashina, 1986; Bodana and Rao, 1990; Nadathur et al., 1994). *L. bulgaricus* was found to prevent 1, 2-dimethylhydrazine (DMH) induced DNA breaks in the colon of rats, whereas *S. thermophilus* was not effective (Wollowski et al., 1999). Treatment of mice with *L. casei* resulted in a reduction of induced by MNNG DNA damage in gastric and colonic mucosal cells. The percentage of cells remaining intact, were distinctly higher in the lactobacilli treated group (68 +/- 19) than in the group receiving only MNNG (45 +/- 17) (Pool-Zobel et al., 1993). *L. acidophilus*, *L. gasseri*, *L. confuses*, *S. thermophilus*, *Bifidobacterium breve* and *Bifidobacterium longum* were antigenotoxic toward MNNG and DMH (Pool-Zobel et al., 1996). The strain of *L. bulgaricus*, known as the "I. Bogdanov strain tumoronecroticance 51 ("LB-51"ATCC 21815) was reported to possess antitumor effects in vivo on transplanted tumors in mice (Sarcoma 180, Ehrlich's solid carcinoma, melanoma, melanosarcoma B-16, plastocystoma MOPS 315, adenocarcinoma ACATOL) (Popova et al., 1993).

The involvement of multiple mechanisms and factors in the prevention of carcinogenesis by probiotics has been suggested:

- (i) Dose dependent antimutagenicity of probiotics was found by Pool-Zobel et al., 1996. The authors observed that  $10^{10}$  viable probiotic cells / kg body weight of rat were antigenotoxic, while one half of this dose resulted in a loss of protective activity.
- (ii) The antimutagenicity of probiotic bacteria was reported to be growth related. Wollowski et al., 2001, suggested that in the linear growth phase, a profound increase in antimutagenic activity occurred, which decreased in the stationary growth phase.
- (iii) In the process of fermentation the probiotic bacteria form short-chain fatty acids that lead to a decrease in the pH of the colon content, associated with a reduced incidence of colon cancer. Depending on the nature, quantity and the fermentability of the indigestible polysaccharides reaching the colon, the relation of short-chain fatty acids acetate, propionate and butyrate can vary. Butyrate may directly enhance cell proliferation in normal cells and apoptosis in transformed cells (Wollowski et al., 2001).
- (iv) Peptides released from milk proteins, mainly caseins, might be responsible for the antimutagenic activity of fermented milks (Matar et al., 1997).
- (v) Probiotic bacteria were shown to decrease the activity of nitroreductase and  $\beta$ -glucuronidase (bacterial enzymes promoting production of mutagenic substances in the colon). Furthermore, probiotic ingestion was shown to stimulate cytochrome P450 activity and glutathione transferase activity in colon cells. The latter is an important enzyme involved in detoxification (Wollowski et al., 2001).
- (vi) Probiotic bacteria were reported to bind mutagenic compounds in the intestinal lumen that correlated with decreased concentration of mutagens in the urine and faeces (Orrhage et al. 1994, and later Hirayama and Rafter, 2000).
- (vii) Yogurt may exert antitumor activity by enhancement of regulatory immune mechanisms, mediated by a larger number of IgA, TNF- $\alpha$  and IL-10 cytokines that favour the apoptosis mechanisms and immune down regulation (Perdigon et al., 2002).

#### **1.1.2.6 Probiotic bacteria in diarrhoea**

One of the main applications of probiotic bacteria has been the treatment and prevention of diarrhoea: acute antibiotic-associated diarrhoea, including severe

intestinal infections such as *Clostridium difficile*; rotavirus diarrhoea in premature infants, newborns and children; nosocomial diarrhoea.

In 1987 Gorbach et al. reported that LGG was efficacious in terminating relapsing colitis due to *C. difficile*. Other studies (Arvola et al., 1999; Vanderhoof et al., 1999) reported beneficial co-administration of LGG ( $10^9$  to  $10^{10}$ ) and antibiotics to children, aged from 2 months to 12.8 years, for the treatment of acute infections of the upper or lower respiratory tract, urinary tract, soft tissues, or skin. LGG reduced the incidence and duration of diarrhoea, stool frequency, and watery or soft stool consistency. The co-administration of rehydration solution and LGG was an effective treatment for acute rotavirus diarrhoea in young children (1 month to 3 years of age) (Rautanen et al., 1998; Guandalini et al., 2000). Analogically, *L. reuteri* ( $10^{10}$  to  $10^{11}$  cfu) was effective as a therapeutic agent in rotavirus diarrhoea in children (6 to 36 months of age) hospitalised with acute diarrhoea (75% rotavirus). The mean duration of watery diarrhoea after treatment was 1.7 days in the *L. reuteri* group and 2.9 days in the placebo group. On the second day only 26% of the patients receiving *L. reuteri* had watery diarrhoea compared with 81% of those receiving placebo (1997 Shornikova et al.). Supplementation with LGG (Szajewska et al., 2001), *Bifidobacterium bifidum* and *S. thermophilus* (Saavedra et al., 1994) reduced significantly the incidence of nosocomial rotavirus gastroenteritis in infants and children hospitalised for reasons other than diarrhoea. LGG proved to be useful also as a prophylactic measure to control diarrhoea in undernourished children (Oberhelmen et al., 1999). *L. acidophilus* fermented milk appeared to prevent radiotherapy-associated diarrhoea in female patients, suffering from gynaecological malignancies and scheduled for internal and external irradiation of the pelvic area (Salminen et al., 1988). Evaluating the evidence by type of acute diarrhoea Sazawal et al., 2006, suggested that probiotics significantly reduced antibiotic associated diarrhoea by 52%, reduced the risk of traveller's diarrhoea by 8%, and that of acute diarrhoea of diverse causes by 34%. Probiotics reduced the associated risk of acute diarrhoea among children by 57% and by 26% among adults. The protective effect did not vary significantly among the probiotic strains *Saccharomyces boulardii*, *L. rhamnosus* GG, *L. acidophilus*, *L. bulgaricus*, and other strains used alone or in combination of two or more strains.

Several possible mechanisms by which probiotics reduce the duration of diarrhoea have been suggested. Probiotics inhibit the adhesion of rotavirus by modifying the glycosylation state of the receptor in epithelial cells via excreted soluble factors (Freitas

et al., 2003). The presence of probiotics also prevents the disruption of the cytoskeletal proteins in the epithelial cells, caused by the *Escherichia coli*, which leads to improved mucosal barrier function and reverse of the increased the secretion of electrolytes (Resta-Lenert and Barret, 2003).

#### **1.1.2.7 Probiotics in bacterial translocation**

Bacterial translocation is defined as the passage of both viable and non-viable microbes and microbial products such as endotoxin across an even anatomically intact intestinal barrier (Wiest, 2005). The bacteria translocating most readily are those usually classified as facultative intracellular pathogens – i.e. those that are able to survive outside the white blood cells but are also able to resist phagocytic killing. Classic examples are *Salmonella* species and *Listeria monocytogenes*. Particularly aerobic and facultative anaerobic Gram-negative bacteria from the indigenous gastrointestinal flora, including *E. coli*, *K. pneumoniae*, other *Enterobacteriaceae*, *P. aeruginosa*, Enterococci and Streptococci, were found to translocate in high numbers to the mesenteric lymph nodes (MLNs) (Wiest, 2005). Curiously, these species are those most frequently associated with complicating infections in severely ill hospitalized and particularly cirrhotic patients. Although intestinal anaerobic bacteria outnumber aerobic bacteria by 100:1 to 1000:1 (Steffen et al., 1988), anaerobes have been reported to translocate only in extreme circumstances such as athymic, lethally irradiated or severely burnt rodents (Wiest, 2005). Bacterial overgrowth is one of the main factors promoting bacterial translocation. Reduced gastric acidity (e.g. due to use of antacids) and predominantly gastrointestinal dysmotility (Nieuwenhuijs et al., 1998) can promote bacterial overgrowth). Increased intestinal permeability is another permissive factor for bacterial translocation. It has been observed in patients with burns, traumas, following cardiopulmonary bypass, elective or emergency vascular surgery, hemorrhagic shock, jaundice, indomethacine, parenteral endotoxin, as well as in intensive care patients (Wiest, 2005). Today it is accepted that many septic complications after injury are due to colonization of the oropharynx, the tracheobronchial tree, and the upper gastrointestinal tract or to translocation of potentially pathogenic microorganisms in the lower gastrointestinal tract. Among organ donors with a median hospital stay of only 1.9 days (range from 6 hours to 8 days) bacterial cultures were positive in 67% most often in mesenteric lymph nodes, lung, liver and spleen. Endotoxin was found in

abdominal fluids in approximately 50% of the cases, in peripheral blood in 20%, and in portal blood in 10% - before any changes were evident in the intestinal wall. Increased intestinal permeability was described as early as 16 to 24 hours after moderate burns. The first peak of endotoxin occurred in the blood of patients about 12 hours after burns (Bengmark and Gianotti, 1996).

Selective gut decontamination has been used to prevent infection by eradicating the potential pathogens, while preserving the normal anaerobic flora. It is performed usually with non-absorbable antibiotics, such as gentamycin, tobramycin, polymyxine, amphotericin B or cefotaxim and its major targets are *Enterobacter*, *Pseudomonas*, *Acetobacter* and yeast (Nathens et al., 1999; Bengmark, 2003).

Another concept to decrease bacterial translocation is by using probiotics such as lactobacilli. Lactobacilli may play an important role in the preservation of the natural biological equilibrium of the intestinal tract and the growth modulation of the other groups of bacteria. Promising results have been observed in treatment of critically ill patients with a combination of pre- and probiotics. Pancreatic necrosis and infections are strong determinants of organ failure and poor outcome in severe acute pancreatitis (Isenmann et al., 1999). Olah et al., 2002 have reported that supplementary *L. plantarum* ( $10^9$  cfu) together with a substrate of oat fiber was effective in reducing pancreatic sepsis and the number of surgical interventions. Infected pancreatic necrosis and abscesses occurred in 1 of 22 patients in treatment group, compared with 7 of 23 in the control group.

Bacterial and fungal infections occur after liver transplantation during the first post-operative month in up to 85% of cases despite of extensive antibiotic treatment and selective digestive tract decontamination. The incidence of postoperative infections after liver transplantation in 95 patients (Rayes et al., 2002) was compared among three different groups, all supplied with early enteral nutrition: (a) a standard formula plus selective bowel decontamination, (b) fiber-containing formula plus living *L. plantarum*, and (c) fibre-containing formula plus heat killed *L. plantarum*. The patients who received living lactobacilli plus fiber developed significantly fewer bacterial infections (13%) than the patients with selective bowel decontamination (48%). Three years later a further improved outcome after liver transplantation was reported using early enteral nutrition supplemented with a mixture of four LAB ( $10^{10}$  *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *L. paracasei* subsp. *paracasei* and *L. plantarum*) and four fibers (2.5g of each betaglucan, inulin, pectin and resistant starch, totally 10g/dose) or

fibers only. The incidence of post-operative bacterial infections was reduced to 48% with fibers and 3% with lactobacilli and fibers (Rayes et al., 2005).

#### **1.1.2.8 Probiotics in wound infection**

Topical application of probiotic bacteria on the skin and around all perforations of the skin by foreign materials such as tubes, drains, tracheostomies etc. is receiving increasing interest (Bengmark, 2003). Moreover, the use of lactobacilli could be of particular interest for treatment of burns. Local treatment with *L. plantarum* of infected with *P. aeruginosa* burn wounds in a burned-mouse model demonstrated inhibition of *P. aeruginosa*, improvement in tissue repair, enhanced phagocytosis and a decrease in apoptosis (Valdez et al., 2005). Burned patients (with infected and non-infected second and third degree burns) treated with *Lactobacillus plantarum* cultures showed the same recovery as the ones treated with silver sulphadiazine (Peral et al., 2009). Another proof of the anti-infective role of certain probiotic bacteria in the healing process of wounds can be found in the study of Nikitenko from 2007. The presence of *Bacillus subtilis* in the wounds of patients following surgery for open or closed limb fractures reduced the possibility of suppuration.

#### **1.1.3 Probiotics as live vaccine delivery vehicles**

The potential of lactic acid bacteria to deliver protective antigens at different mucosal surfaces has been investigated. Three lactic acid bacterial hosts have been evaluated as potential vaccine vehicles: *Lactococcus lactis*, a cheese starter strain, as a prototype of non-colonising strain; lactobacilli which are able to colonise given body cavities; and *Streptococcus gordonii*, an oral commensal bacterium of human origin (Mercenier et al., 2000).

Successful immunization of mice resulting in a protective immune response with a lactic acid bacterium in the absence of additional adjuvants was achieved using the non-toxic C fragment of tetanus in the cytoplasm of *Lactococcus lactis* (Wells et al., 1993). This positive result initiated a series of experiments, in which TTFC was invariably used as the model antigen. Many variables (e.g. strain dependence, expression levels, cellular location of expression and immunization schemes) were tested. Experiments showed that recombinant *L. plantarum*, expressing TTFC was equally capable of

eliciting an immune response, irrespective of the site of expression (intracellularly, anchored to the cell wall or secreted) however with different response levels. While cell-surface presentation required lower antigen doses to be immunogenic, the highest IgG serum antibody titres were obtained with the strain producing large amounts of TTFC in the cytoplasm (Reveneau et al., 2002). A comparison between *L. plantarum* and *L. casei*, in which TTFC was expressed either as an intracellular or a surface exposed protein, showed that regardless of the route of administration (oral or intranasal), responses were always higher when *L. plantarum* was used as delivery system (Shaw et al., 2000). These results confirm that *Lactobacillus* strains can differentially influence the immune system.

Independent studies showed that TTFC – specific response following intranasal as well as oral immunization could be obtained with *L. plantarum* expressing TTFC intracellularly (Reveneau et al., 2002; Shaw et al., 2000). Endpoint titres were higher following intranasal immunizations than following oral immunizations. To determine whether these immunizations require live bacteria, a comparison was made between mice that were immunized intranasally with either live or mitomycin-C treated bacteria (Granette et al., 2001). This reduced the viable count from  $10^9$  to  $0.5 \times 10^5$  colony forming units (cfu). No differences were observed in total serum TTFC-specific IgG level but protection levels as determined by neutralizing tetanus antibody levels, were eightfold lower with mitomycin-C treated bacteria than with live bacteria.

In parallel to this work, Steidler et al., 1998, demonstrated that host immune responses could be enhanced by co-delivery of IL-2 and IL-6 and TTFC. The approach of delivering cytokines with known modulatory properties was continued by the construction of recombinant *L. lactis* strains secreting murine IL-10 (Steidler et al., 2000). The authors showed that these strains were able to prevent or treat inflammation in 2 murine colitis models. Notably this effect was obtained with much lower doses of IL-10 than those required when the cytokine was used as free polypeptide. Steidler et al., 2003 further constructed a safe (no antibioresistance marker and chromosomally integrated transgene) biologically contained strain secreting human IL-10 and a human intervention trial (targeting IBD) with this strain has been conducted (Baat et al., 2006).

Lactobacilli are currently under investigation not only in active vaccination but also in passive vaccination. Passive vaccination requires the delivery of pathogen- or toxin-neutralising agents – most commonly immunoglobulins. Complete immunoglobulins

are complex molecules that are difficult to produce in bacteria and strategies are therefore being developed for secretion and cell-wall anchoring of single-chain polypeptides, which comprise only the binding domain of the immunoglobulins. The value of this technique was shown in the study of Krüger et al., 2002. Vectors encoding a single-chain Fv (scFv) antibody fragment, which recognizes the streptococcal antigen I/II (SAI/II) adhesion molecule of *Streptococcus mutans*, were constructed and expressed in *L. zae*. The scFv antibody fragments expressing bacteria agglutinated SAI/II-expressing *S. mutans* in vitro and reduced *S. mutans* bacterial counts and caries after administration to a rat model of dental carries. Chang et al., 2003, have engineered natural human vaginal isolates of *L. jensenii* to secrete two-domain CD4 (2D CD4) proteins. The secreted 2D CD4 recognized a conformation-dependent anti-CD4 antibody and bound HIV type 1. *Lactobacillus*-derived 2D CD4 inhibited HIV-1 entry into target cells in a dose-dependent manner.

As described above, there are fundamental differences between various probiotic strains. The expected clinical effects from the use of different probiotic strains are most various and conclusions cannot be made from one strain to another. Unravelling the mechanisms underlying the complex interactions among probiotic and pathogenic bacteria as well as among probiotic bacteria and host microflora, epithelial cells and immune cells would permit better understanding of the probiotic effect.

## **1.2 Examples of delivery systems containing lactobacilli and other probiotic bacteria**

The reliability of probiotic strains in terms of quality and functional properties is dose-dependent. The supply of a probiotic organism in a number of  $10^7$  or lower to the site of administration is generally regarded as being too small for significant probiotic effects to be expected (Bengmark, 2003). Consequently, the ability of probiotic organisms to withstand the adverse conditions, encountered in industrial processes, and the natural barriers of the host, has become essential for the development of probiotic applications such as bacteriotherapy, live vaccines and probiotic foods. Stability of probiotic organisms can be demonstrated only if they still show metabolic activity after storage and intake by a host. Based on the biological limitations of microorganisms originating from their genetically determined metabolic peculiarities and their



phenotypes, two ways for manufacturing of probiotic products can be used (Viernstein et al., 2005; Alpas et al., 2000; Niemela et al., 1994). One way is the direct use of fresh, concentrated, refrigerated or frozen probiotic cultures (e.g. yogurts, ice creams), which has the advantage of very limited loss of viability, but the limit of a short storage time (four to six weeks). The other way relies on the principle of transforming bacteria in a dormant state by drying techniques (e.g. freeze-drying). When freeze-drying is applied to microorganisms the cellular function is suspended temporarily because it is cut off from the metabolic activities that are mediated by the action of water (Souzu, 2004). This permits their preservation for a longer period of time and gives them further flexibility for incorporation into delivery systems. Alternative drying processes with lower costs, such as spray drying, fluidized bed drying and vacuum drying have been considered (Santivarangkna et al., 2007a). A major drawback of cellular dehydration is related to a higher risk of physical cellular damage (Viernstein et al., 2005).

## **1.2.1 Drying processes**

### **1.2.1.1 Spray drying**

In heat drying processes like spray drying, probiotic cultures are atomized into a flow of hot air, e.g. 150-200°C. Inactivation of cells can occur due to both high temperature and dehydration. Temperature inactivation of an atomized cell suspension at the beginning (constant drying rate period) is limited by the evaporative cooling effect. At the following stage (falling drying rate period), the temperature inactivation of the cells increases depending on the drying parameters such as inlet/outlet air temperatures, residence time and feed rate. The outlet air temperature at which the product leaves the drying chamber is believed to have a major impact on bacterial viability (Santivarangkna et al., 2007a, 2008). It was found that below 64°C damaging sites are structures contained in or making up the cell membrane. For temperatures of 65°C and above, ribosome and/or protein denaturation as well as cell wall damage may be responsible for the thermal death (Teixeira et al., 1997).

### **1.2.1.2 Fluidized bed drying**

Heat inactivation can be minimized and easier controlled by using relatively low air temperatures in fluidized bed drying. Cells must be entrapped or encapsulated in support materials such as skim milk, potato starch, alginate and casein as only

granulable materials can be dried (Santivarangkna et al., 2007a). However, the water activity of the support material can pose an osmotic shock, the severity of which determines the bacterial viability. When *L. plantarum* cells were mixed with extremely desiccated casein powder ( $a_w \leq 0.1$ ), viability of the mix did not exceed 2.5% (Mille et al., 2004).

### **1.2.1.3 Vacuum drying**

Vacuum drying is a suitable process for heat sensitive materials. Since the drying operates under vacuum, moisture can be removed from the materials at a low temperature. For example while the boiling point of water at 100 kPa (1 atm) is 100°C, at 1 kPa it is approximately 8°C. Moreover, the oxidation reactions during drying can be minimized for oxygen sensitive bacteria (Santivarangkna et al., 2007a). The process, however, was reported to result in lower viability of *L. acidophilus* compared to freeze-drying (Conrad et al., 2000). Investigating the mode of inactivation of *L. helveticus* due to vacuum drying, Santivarangkna et al., 2007b, found that cell injuries were attributed to damage in the cell membrane and wall.

### **1.2.1.4 Freeze drying**

Freezing and drying, also called lyophilization, is the appropriate choice for production of dry but viable cells of most microorganisms. Cellular damage of microorganisms is considered to be accumulated result, which might have been initiated during the freezing stage, but detected after dehydration. Detrimental chemical reactions, which could be induced in the state of lower water activity, might continue through the early drying stage to the storage period (Souzu, 2004).

Bacterial tolerance to freezing and drying is related to cell growth phases and growth environments. Generally, logarithmically growing cells are very susceptible to freezing and freeze-drying damage. For example, *Escherichia coli* cells grown at two different temperature ranges showed an increasing tolerance to freeze-thawing and freeze-drying when cell growth approached its stationary phase (Table 1.3). It is related to increasing structural stability of the cellular membrane accordingly to the cell growth phases (Souzu, 2004).

Table 1.3 Viability of freeze-thawed or freeze-dried *E. coli* cells cultivated at different temperatures and differing growth phases.

Cultivations		Survival of Cells		
		Freeze-thawed		Freeze-dried
°C	H	-5°C	-30°C	
	2 *	4	3.8	4
37	3 **	52.3	50.4	24.9
	5 ***	93.9	92.5	38.6
	8 *	6.2	5.7	0.2
17	20 **	52.2	35.5	8.2
	48 ***	82.8	51.4	47.8

(H) Incubation time (\*) Cells growing in logarithmic; (\*\*) Early stationary; (\*\*\*) Stationary phase (after Souzu, 2004)

The growth temperature as well as the sugar substrates in the growth medium can also affect the tolerance of cells to freeze-drying and their survival during storage. Incubation of *E. coli* cultures at 42°C promoted significantly higher survival rate of the organism during freeze-drying, compared to incubation at 30°C (Souzu, 2004). The lowest decrease in viability of *L. bulgaricus* cells after freeze-drying was obtained when the organism was grown in the presence of mannose. Moreover, *L. bulgaricus* clearly survived better during storage when cells were grown in the presence of fructose, lactose or mannose rather than glucose (the standard sugar in the growth medium) (Carvalho et al., 2004).

Cell concentration of the final suspension before freeze-drying has a significant effect on the survival rate. A high concentration of the cells prevents suitable heat conductivity in the freeze-drying process and thus hinders the effective sublimation of ice crystals and also desorption of unfreezable water molecules in the drying process through formation of a thick interstitial network in the specimen. On the other hand, if cell suspensions are too low concentrated, the cell viability might be lowered through lack of the protective effects generated by mutual contact of the cells. Besides, cells can be dispersed by the stream of the water vapour formed during ice crystal sublimation because they lack effective networks. As a result many cells will be lost in the process (Souzu, 2004).  $10^9$ - $10^{10}$ cfu/ml was found to be an optimal bacterial cell concentration resulting in the highest survivability during freeze-drying in the studies of Costa et al., 2000 and Palmfeldt et al., 2003.  $10^{12}$  cfu/ml was reported to be too concentrated for freeze-drying of *L. bulgaricus* and *S. thermophilus* (Bozoglu et al., 1987).

Cellular damage is thought to be caused by increasing concentration of solutes or growth of ice crystals in the cells during extracellular and intracellular freezing. The cooling rate is one of the most important factors during freezing. Since the cytoplasm of a cell generally remains unfrozen at  $-10^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$ , extracellular ice crystals usually form first and the solutes will be concentrated in a smaller volume of unfrozen water. Cells will lose water to maintain the osmotic equilibrium between the inside and the outside of cells if the rate of freezing is very low. Consequently the alteration in the intra- and extracellular solutions produced by ice formation outside the cells results in cell dehydration. For this reason, the slow freezing injury is referred to solution effect injury (Mazur 1970). In contrast, if the cooling rate is so high that cells are not able to lose water rapidly enough to maintain the osmotic equilibrium, intracellular ice formation will occur. Different views on intracellular ice formation have been reviewed by Santivarangkna et al., 2008. The phenomenon was hypothesised to result from damages or defects developing in cell membranes. Consequently, an optimal cooling rate which to be low enough to avoid intracellular ice formation but high enough to minimize solution effects could to prevent cellular damage. Moreover, hydrophilic and cell membrane-penetrable materials might be anticipated as protective substances. Glycerol, dimethylsulfoxide and small molecular weight sugars have been described to repress ice crystal generation or retard its growth in solution, during slow and rapid freezing. No ice crystals have been observed in red blood cells or in yeast cells, in the presence of 30% glycerol, and their viability after thaw was almost 100% (Souzu, 2004).

Another factor in maintaining cell viability would be to stabilize the cell membrane itself. The cell membrane is the principal site of inactivation during freezing as well as during dehydration. In normal membranes phospholipids are hydrated and adsorb various amounts of water depending on the type of phospholipid. The removal of the water increases opportunities of van der Waals' interaction among the hydrocarbon chains. In membranes in which the relative water content is less than 0.25 g  $\text{H}_2\text{O}$  per g dry material, it may cause large lateral compressive stresses in the plane of membrane and phospholipids are likely to undergo phase transition (Wolfe, 1987), e.g. from liquid crystalline ( $L_{\alpha}$ ) to gel phase ( $L_{\beta}$ ). Consequently at a physiological temperature where lipids are usually fluid and molten, the lipids may begin changing to gel phase during drying. In other words, there is a corresponding gradual increase in the membrane phase transition temperature ( $T_m$ , the midpoint of a transition temperature range) as the water

content decreases. This increase in  $T_m$  generally becomes detectable at water contents less than approximately 0.2 g H<sub>2</sub>O per g dry material (Santivarangkna et al., 2007a). In probiotic cultures,  $T_m$  of *L. plantarum* was reported to shift from 4°C in hydrated cells to 20°C in dried cells (Linders et al., 1997) and from 35 to 40°C in *L. bulgaricus* (Oldenhof et al., 2005). Cell membranes stability is increasing through the contribution of hydrogen bonding and hydrophobic bonding between membrane materials and water molecules in the system. Protective substances for freeze-drying with hydrogen bond forming ability are classified into low molecular weight compounds such as amino acids, organic acids, sugars, and sugar alcohols, and high weight substances such as proteins, polysugars, polyvinylpyrrolidone, and synthetic polymers (Leslie et al., 1995; Carvalho et al., 2002; Linders et al., 1997; Souzu, 2004; Oldenhof et al., 2005). Table 1.4 summarizes substances used in varying combinations as cryoprotectants for probiotics.

Table 1.4 Cryoprotectants for probiotic microorganisms.

Adonitol	Skim milk powder	Dimethylsulphoxide	Polyethyleneglycol
Alpha-tocopherol	Maltodextrin	Gelatine	Sucrose
Beta-glycerophosphate	Malt extract	Glucose	Sorbitol
Bovine albumin	Mannitol	Glycerol	Trehalose
Calcium alginate	Milk fat	Glycogen	Tween
Calcium carbonate	m-Inositol	Lactose	Xanthan gum
Casein hydrolysate	Na-alginate	L-ascorbic acid	Yeast extract
Carnithine	Na-glutamate	L-asparagine	Whey powder
Dextran	Pectin	L-cystein	

(after Viernstein et al., 2005)

Determination of the endpoint of the process of freeze-drying is another critical aspect. Higher moisture content in the probiotic products has damaging effect during storage, but overdrying of living cells results not only in impairment of cellular constituents but also triggers chemical reactions including aminocarbonyl reaction, free radical generation, and unbalanced metabolism (Souzu et al., 2004). Zayed et al., 2004 showed that a residual water content of 2.8 to about 5.6% improved survival rate of lyophilized *L. salivarius* during storage. On the other hand, removing water from the dried cells (0.0% moisture content), as well as higher moisture content (8.8%) resulted in viability decrease of 44 and 17% in 1 week and 72 and 92% in 7 weeks, respectively.

Carefully handled freeze-dried products can theoretically withstand storage, over a wide temperature range. However, oxidative reactions, enzymatic evolution, and chemical degradation containing fatty acid oxidation are highly temperature dependent,

especially at higher temperatures (Souzu, 2004). Thus storage in a moderate temperature range, most reliable between 5 and 10°C, will improve the cell viability over a longer storage period. Light and oxygen are also detrimental (Kandler and Weiss, 1986; Viernstein et al., 2005).

### **1.2.2 Powders**

The described drying techniques are used to obtain probiotic powders, which on the other hand can be used for sachet fillings and hard gelatine capsule fillings. Other vehicle forms for probiotic powders are pellets and tablets. It should be taken into consideration that each further processing step is related to further bacterial death.

### **1.2.3 Capsules**

Hard (gelatine) capsules are an ideal dosage system for powders and granules. The capsule filling process is a process with low physical impact on the bacterial material. Film coating may be applied to the capsules for protection against gastric juice or for controlled release. Huyghebaert et al., 2004 suggested the usage of enteric pre-coated capsules to overcome problems of destabilization of heat and moisture sensitive materials such as lyophilized biomaterials (peptides, proteins, bacteria).

### **1.2.4 Tablets**

Tablets are still the most common pharmaceutical dosage form, which relative to other dosage types are inexpensive and could be therefore of interest for the production of probiotic bacteria loaded tablets. In general, tableting is not the ideal process for products comprising viable bacteria, because the excess of forces used to compact a powdery or granulated pre-mixture cause physical damage into the dry cell walls and membranes (Viernstein et al., 2005). Chan and Zhang, 2002, examined the effect of compression pressure (from 0 to 180 MPa) on the viability and sublethal injuries of *L. acidophilus*. The authors found that there was no significant loss in cell viability up to the pressure of 30 MPa. However, compaction even at these low pressures still caused some damage to the wall of the cells. When the compression pressure was increased from 30 to 90 MPa, there was a gradual increase in loss of cell viability. When the

pressure exceeded 90 MPa, damage not only occurred to the wall of the cells but also to their membranes and cell viability decreased almost linearly with the compression pressure. Consequently in order to minimize the cell loss by compression, the pressure should be as low as possible but simultaneously enough to ensure tablet rigidity for further handling.

It should be taken in consideration that different strains exhibit different tolerance to pressure. Maggi et al., 2000 demonstrated that three out of ten strains, including *L. salivarius* and *L. gasseri*, maintained high viability during tableting. Formulation components can play an important role in microorganism inactivation or protection. Blair et al., 1991 found that the type of consolidation during compaction (plastic or brittle fracture) influenced survival of *Staphylococcus aureus* and *Enterobacter cloacae*. Plastic deforming microcrystalline cellulose (Avicel PH-101) caused the greatest bacterial lethality at low compression forces compared to lactose monohydrate and maize starch. Different studies showed that skim milk powder, sodium alginate, hydroxypropylmethylcellulose acetate succinate (HPMC-AS) and HPMC-phtalate can be used as matrix-forming excipients providing resistance of microorganisms (*L. acidophilus*, *L. fermentum*) to compression, gastric juice and during storage (Stadler and Viernstein, 2003; Klayraung et al., 2009). Inclusion of probiotic-specific substrates known as prebiotics, could be a strategy to improve survival at the site of administration. A prebiotic is defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and / or activity of probiotic bacteria (Gibson and Roberfroid, 1995). Inulin and its hydrolysate, oligofructose are among the most common prebiotics (Roberfroid, 2007).

### **1.2.5 Pellets via extrusion / spheronisation processing**

From a current standpoint extrusion / spheronisation is not considered an optimal option for the stabilisation of bacteria (Viernstein et al., 2005). The physical impact on the biological materials, including rise in temperature and mechanical stresses: compaction pressure and shear forces (Ghebre-Sellassie, 1989; Vervaet et al., 1995), can exceed the limits of cell survival. Another important factor affecting the viability, particularly when fresh bacterial culture is used, is the progressive decrease of water content. Drying of bacteria starts with the mixing of bacteria with powders promoting high osmotic pressure (Mille et al., 2004) and continues during extrusion and

spheronisation (Ghebre-Sellassie, 1989; Vervaet et al., 1995). When osmotic shock is applied via powders, the osmotic gradient induces water exit, but little or no solute can penetrate into the cell. The volume variation of the cell can result in cell death (Mille et al., 2004). A strain-dependent survivability has been documented in literature. Kim et al., 1988, carried out extrusion / spheronisation experiments using lactic acid bacteria incorporated in a medium of skim milk, food grade cellulose and glycerol. The authors showed that cell morphology could influence the survival rates of different bacterial cultures. They reported no loss of viability of *Streptococcus cremoris* and *Pediococcus acidilactici* (both cocci) in pellets after 1 week storage at room temperature, but a loss of viability of more than 1 log unit of *L. plantarum* (rod-shaped) in pellets stored at the same conditions. Storage at 4°C for a period of 35 to 76 days resulted in stable viability of all organisms ( $10^9$ cfu/g for *S. cremoris*,  $10^{11}$ cfu/g for *L. plantarum* and *P. acidilactici*). Contrary to Kim et al., 1988, Huyghebaert et al., 2005a, reported a detrimental effect of extrusion / spheronisation processing on *Lactococcus lactis*. The authors used *L. lactis* culture as a granulation fluid of microcrystalline cellulose containing sodium carboxymethyl cellulose, low substituted hydroxypropyl cellulose and starch. They observed a 2 log viability decrease of the organism after pelletisation and drying and a further 2 log decrease in 1 week of storage at room temperature and low relative humidity. The authors suggested that the shear developed during granulation and mainly during extrusion / spheronisation combined with rise in temperature (30-40°C) could be responsible for the drop of viability and sublethal injury which accumulated later in drying and storage.

### 1.2.6 Coated solid particles

A protective coating around particles loaded with microorganisms is an approach for stabilization. However, the coating temperatures should not exceed the bacterial growth temperature range and the solvent for coating materials can only be water. Furthermore, the hygroscopic nature of the dry particles will result in the uptake of moisture from the coating suspensions. Therefore water removal is essential to avoid degradation processes in the solid particles (Viernstein et al., 2005). In 2005 Huyghebaert et al., (b), evaluated in vitro the enteric properties of different polymers for coating of *Lactococcus lactis* layered pellets. Aqoat AS-HF and Eudragit S provided good gastro-resistant properties. However, coated pellets had to be cured at elevated temperatures (60°C),



which made the polymers improper for the coating of the temperature sensitive organisms. On the other hand, Eudragit L30D-55 and a mixture of Eudragit FS30D / L30D-55 dissolved at a pH lower than 6.8 and consequently bacteria would be subjected to the bile salts present in the jejunum. In contrast Eudragit FS30D dissolved at pH 7.2-7.4 and consequently the bacteria would only be released in the most distal part of the ileum. Further investigation of the effect of Eudragit L30D-55 and Eudragit FS30D on the viability of *L. lactis* showed a better survivability of the organism during processing and in simulated gastric acid when coated with Eudragit FS30D (Huyghebaert et al., 2005c).

### **1.2.7 Immobilisation techniques**

Cell immobilisation approaches represent an alternative strategy in which cells are immobilised within biocompatible polymers and have been shown to offer many advantages for biomass and metabolite productions compared with free-cell (FC) systems such as: high cell density, retention of plasmid bearing cells, improved resistance to contamination, stimulation of production and secretion of secondary metabolites and physical and chemical protection of the cells (Scannell et al., 2000). Most widely the immobilisation techniques rely on entrapment of cells within porous polymeric matrix such as alginate, chitosan,  $\kappa$ -carageenan, gellan, agarose and gelatine (Lacroix et al., 2005). Another method is the adhesion or adsorption to a preformed matrix, such as sintered glass, porous glass, ceramic particles, silicon rubber (Gonçalves et al., 1992; Bergmaier et al., 2003), that imitates what takes place in a natural environment where cells are almost always associated with surfaces and grow in biofilm (Dunne, 2002).

Cells produced by immobilisation technology especially when combined with continuous cultures exhibit changes in growth, physiology as well as morphology compared with the cells produced during conventional free cell cultures. Cachon et al., 1998, observed differences in cell physiology (redox state, enzymatic pool and intracellular pH) between free and immobilised *L. lactis*. A shift in the metabolic pathway from homofermentative to heterofermentative has been observed during continuous cultures with immobilised *L. plantarum*, accompanied by morphological changes in immobilised cells from the normal rod shape to the coccoid shape (Krishnan et al., 2001). Moreover, an increased tolerance of immobilised cells (IC) to alcohols,

phenols, antibiotics or quaternary ammonium sanitizers was observed (Lacroix et al., 2005). Trauth et al., 2001, explained the phenomenon of increased cell tolerance of the IC used in continuous fermentations, by possible modification of the cell membrane and physiology, and by cell proximity in a saturated matrix. In colonised beads the cell density inside the gel matrix could reach 400g dry weight / l, or about  $1 \times 10^{12}$  cell/ml (Trauth et al., 2001). However, the cell growth in colonised gels is non-uniform. It is characterised with a high cell density region near the bead surface with a thickness varying from 100 to 400 $\mu$ m (Massen et al., 1994; Lacroix et al., 2005). The growth inhibition in the central part of the gel bead may be explained by the combined effect of high product concentration and low pH, promoting the undissociated form of lactic acid, which has been associated with inhibition of lactic acid bacteria (Massen et al., 1994). Massen et al., 1994, observed a pH decline from the surface (pH 4.35) to the centre (pH 3.8) of the gel bead. Actually, the development of steep inhibitory product, pH and biomass gradients can induce a non-specific stress adaptation (Lacroix et al., 2005).

Bacterial immobilisation has also been shown to improve bacterial viability during storage and protect bacteria from adverse processes, such as freezing and freeze-drying and hostile environments, such as gastric juices. *Bifidobacterium longum* strains encapsulated in  $\kappa$ -carrageenan microcapsules showed no difference in the cell populations during refrigerated storage of 30 days, while non-encapsulated bifidobacteria showed a decline of 0.54 and 0.63 log units for the same period of time. A higher stability was reported for *L. bulgaricus* cells immobilised in alginate beads compared with FC during storage of frozen dairy desserts (90% vs 40% survival) (Sheu et al. 1993). Protective effect of alginate bead microenvironment to *L. plantarum* during freeze-drying was reported by Kearney et al., 1990. In this study the incorporation of cryoprotective agents (adonitol and skim milk) in the alginate solution increased the protective effects of immobilisation and gave survival rates as high as 85.9% compared with 64.6% for FC lyophilized with the same cryoprotectants. Cui et al., 2000, prepared *Bifidobacterium bifidum*-loaded alginate microparticles. The obtained microcapsules were further suspended in poly-*l*-lysine solution to cross-link the particles. Alginate as well as poly-*l*-lysine treatment enhanced highly the survival of bifidobacteria in simulated gastric environment (ca.  $>10^8$  vs.  $<10^3$  cfu/g). Screening of different microencapsulation conditions of *L. acidophilus* showed that highest protection of the organism at low pH (pH 2) and high bile concentration (1%) was obtained with 1.8% (w/v) alginate,  $10^9$  cfu/ml, 30 min hardening in 0.1 CaCl<sub>2</sub> and capsule size 450  $\mu$ m

(Chandramouli et al., 2004). Sun and Griffiths, 2000, reported a protective effect of immobilization of *Bifidobacterium infantis* into gellan-xanthan beads to simulated gastric juices. At pH 2.5 the viable count of free cells dropped from  $1.23 \times 10^9$  to an undetectable level (<10 cfu/ml) in 30 min, while the viable count of the immobilised cells decreased by only 0.67 log cycle in the same time period. Microencapsulation of *Bifidobacterium longum* B6 and *Bifidobacterium infantis* carried out by spray drying the cell suspension containing the test organism and 10% of carrier material of gelatine, soluble starch, skim milk or gum arabic, had also protective effect on bifidobacteria to simulated gastric and bile environment (Lian et al., 2003).

Finally, the application of IC technology could be advantageous to conventional technologies by development of products retaining high concentrations of metabolic active bacteria with prolonged viability and enhanced tolerance to various stresses.

### **1.3 Aim of the Thesis**

The aim of the Thesis was the incorporation of *Lactobacillus* spp. into different dosage forms to evaluate their viability and antibacterial activity against multi-resistant bacteria.

### **1.4 Organization of the Thesis**

The first part of the experimental work aimed to provide evidence of the viability of *Lactobacillus* spp. (*L. plantarum*, *L. rhamnosus* GG and *L. bulgaricus*) and a spore form of *B. subtilis* from non-processed bacteria to coated dosage forms (i.e., mini-tablets, pellets and their coated forms). The development of the dosage forms involved: evaluation of processing techniques (freeze-drying, tableting, pelletization and coating) and excipients (skim milk, microcrystalline cellulose, inulin ST and HP, lactose, Eudragit FS30-D) on the bacterial viability and comparison of the relative viability among the bacterial strains, regarding their processibility and stability throughout storage.

The second part of the experimental work aimed to produce calcium alginate beads able to deliver *Lactobacillus* spp. (*L. plantarum*, *L. rhamnosus* GG, *L. bulgaricus* and *L. lactis*) with preserved viability and antibacterial activity. Four types of beads,

containing entrapped (E), surface and entrapped (ES), surface (S) and concentrated surface and entrapped lactobacilli (C<sub>ES</sub>) were prepared and physically characterised. The antibacterial activity of lactobacilli cultures before and after immobilization and freeze-drying and throughout storage was studied in relationship to the viable number of lactobacilli. Multi-resistant clinical isolates (methicillin-resistant *Staphylococcus aureus*, vancomycine-resistant *Enterococcus faecalis*, VIM-2-metallo- $\beta$ -lactamase producing *Pseudomonas aeruginosa* and CTX-M-15- $\beta$ -lactamase producing strains: *Escherichia coli* and *Klebsiella pneumoniae*) were used as indicator strains.

The third part of the experimental work aimed at the development of calcium alginate films, containing *Lactobacillus plantarum* with preserved and stable viability and antibacterial activity suitable for topical administration. Calcium alginate films containing both entrapped and surface lactobacilli were produced with different calcium concentrations. Films were physically characterized for mechanical, adhesive properties and lactobacilli release. The viability and antibacterial activity of *L. plantarum* was studied before and after processing and during 6 months of storage. A multi-resistant clinical isolate, VIM-2-metallo- $\beta$ -lactamase producing *Pseudomonas aeruginosa*, was used as the indicator strain. To test the *in vivo* activity of the immobilized lactobacilli, a rat model of burn injury was used in which burned skin contaminated with *P. aeruginosa* was exposed to *L. plantarum* loaded films.

The parts mentioned were designed based on the state-of-the-art presented in the first chapter of the thesis, that is divided into two parts, one related to the identification of the advantages of using the bacteria in routine applications and a second part related to the most relevant dosage forms to deliver *Lactobacillus* bacteria to humans.

The last chapter (chapter 5) summarizes the main aspects that have been learnt from all the work, as concluding remarks.

## **CHAPTER 2**

### **EVALUATION OF THE VIABILITY OF *LACTOBACILLUS* SPP. AFTER THE PRODUCTION OF DIFFERENT SOLID DOSAGE FORMS**

## 2.1 Introduction

The continuous consumption of probiotics has been claimed to promote the health condition of individuals (Halpern et al., 1991). Among a vast number of probiotic microorganisms, strains from genus *Lactobacillus* have been used for centuries. A common problem found in the use of microorganisms is that the number of viable cells determines the probiotic effect and thus, the stability of the microorganisms in probiotic products is critical. Often, the starting point is in the order of  $10^9$  cells per gram of product. However, in storage this number decreases dramatically several orders of magnitude reaching  $10^3 - 10^6$  cfu/g (Temmerman et al., 2003). Other major concern is the decrease of the number of viable cells in the stomach (Toumola et al., 2001).

Different strategies for stabilization of cells have been taken into consideration, namely tableting of freeze-dried bacteria (Maggi et al., 2000, Stadler and Viernstein, 2003), production of pellets (Huyghebaert et al., 2005a) or encapsulation of bacteria (Chan et al., 2005). Some authors have considered coating the dosage forms to observe their disintegration in the intestine (Chen, 2007). Commonality to the works is that cells have to be harvested from the culture media and dried (often by freeze-drying) in protective matrices and, thus, enabling processing, increasing preservation and allowing easy reconstitution of the bacteria at the site of administration (e.g. gastrointestinal tract).

## 2.2 Materials and Methods

### 2.2.1 Materials

The examined probiotic strains were *Lactobacillus plantarum* ATCC 8014, *Lactobacillus rhamnosus* strain GG ATCC 53103, LGG, *Lactobacillus delbrueckii* subsp. *bulgaricus* CIP 101027 and *Bacillus subtilis* ATCC 6633.

Two types of inulin have been used (ST, a standard inulin and HP, a high performance inulin, respectively Raftiline ST and HP, Orafiti, Oreya, Belgium). Other excipients used were microcrystalline cellulose (Avicel PH101, FMC Corporation, Cork, Ireland), lactose monohydrate (Granulac 200, Meggle, Wasserburg, Germany), skim milk (SM, Difco, Detroit, MI), glycerol (Sigma-Aldrich Chemie, Steinheim, Germany), (D+) mannose (Merck, Darmstadt, Germany), glyceryl

monostearate (Sigma-Aldrich Chemie) and poly(meth)acrylate derivative (Eudragit FS30D, a colonic coat, Evonik-Röhm, Darmstadt, Germany).

## **2.2.2 Methods**

### **2.2.2.1 Strains and culture conditions**

Lactobacilli were cultivated overnight on de Man-Rogosa-Sharpe (de Man et al., 1960), (MRS, Biokar Diagnostics, France) agar and in broth at 37°C. *B. subtilis* was incubated on plate count agar (PCA, Biokar Diagnostics, Beauvais, France) at 37°C for two-weeks to produce spores. The two-week old cultures were then concentrated up to 10<sup>7</sup> cfu/ml in sterile distilled water and pasteurized in a water bath at 80°C for 20 min to acquire spore suspensions without vegetative forms.

### **2.2.2.2 Freeze-Drying**

Cells, harvested from overnight broth cultures (20ml) by centrifugation at 4000 rpm for 20 min at 4°C (Jouan CR411, Jouan, Saint Nazaire, France), were washed twice in Ringer's solution and re-suspended in equivalent volumes of reconstituted skim milk (10%) or growth medium (MRS), containing 1% of glycerol or mannose as cryoprotectors. The suspensions were kept for 1h at room temperature prior to freezing at -80°C for 24h (Carvalho et al., 2004). By analogy, the spores harvested by centrifugation were re-suspended in skim milk enriched with 1% glycerol and frozen immediately afterwards. The frozen samples were dried under vacuum at 0.035mbar for 72h at room temperature (25°C) (Christ LDC-1m Alpha 2-4, B. Braun, Biotech International, Melsungen, Germany). Water content and bacterial viability in the freeze-dried powders were evaluated.

### **2.2.2.3 Production of mini-tablets**

Lyophilizates of each bacterial strain were compressed to mini-tablets. The freeze-dried cakes were ground in a mortar alone or together with different excipients in a ratio of 1:1 or 3:1 parts of the compaction aid, either inulin or microcrystalline cellulose (Table 2.1, upper part). The mixtures were compacted in a single punch tableting machine (Korsh, Erweka GmbH, Heusenstamm, Germany) fitted with 2.5 mm diameter

punches and die. The powders were compacted at 1, 2 and 5kN (25.5, 50.9 and 127.3MPa) to produce tablets with an average weight of 6.75mg. The size (n=10), weight (n=10), crushing force (n=5) (European Pharmacopoeia 5, 2.9.8 (European Pharmacopoeia), tensile strength (Fell and Newton, 1970), density (n=10) (helium pycnometry, Accupyc 1330, Micromeritics, Norcross, GA), disintegration time (n=6) (European Pharmacopoeia 5, 2.9.1 (European Pharmacopoeia) and viable number of the mini-tablets were evaluated.

#### **2.2.2.4 Production of pellets**

Microcrystalline cellulose (10g) and lactose (40g) were blended in a planetary mixer (Kenwood-Chief, Kenwood, UK), for 5min. Then, 25g of inulin HP and 25g of skim milk (SM) were added to the mixture and the blending continued for another 5min. The overnight cultures of each *Lactobacillus* strain were tenfold concentrated by centrifugation and re-suspension of the cell pellets in tenfold smaller quantity of physiological saline. The concentrated lactobacilli cultures and *B. subtilis* spore suspension were then used as granulation liquids (30g), gradually added to the mixture of powders (pel 1, Table 2.1, lower part). The wet mass was extruded in a ram extruder (12.5 mm diameter, Lloyd K50, Lloyds Instruments Ltd. Farnham, Hampshire, UK) at 400mm/min at a maximum force of 5kN through a 1 mm diameter die. The extrudates were spheronised in a spheroniser with a cross hatched plate (Caleva 230 spheroniser, Caleva, Sturminster Newton, Dorset UK) for 10 min at 1000 rpm. Pellets were dried in a fluid bed drier (Strea, Niro-Aeromatic, Bubbendorf, Switzerland) at 30°C for 60 min prior to evaluation for size (laboratory test sieve analysis, Retsch, Haan, Germany, for 5 minutes), weight, force required to crush them (pellets were placed between two horizontal jaws, perpendicular to their movement and crushed, CT5, Engineering System, Nottm, UK) (European Pharmacopoeia 5, 2.9.8 (European Pharmacopoeia) disintegration time (European Pharmacopoeia 5, 2.9.1 (European Pharmacopoeia) and density (helium pycnometry). In order to evaluate the effect of increasing the amount of inulin in the pellets, a second formulation for *L. plantarum* only, was considered (pel 2, Table 2.1, lower part).



Table 2.1 Composition of Formulations (in parts).

Formulations of mini-tablets					
	Lyophilizate	Skim milk	Inulin ST	Microcrystalline cellulose	Inulin HP
Tab 1	100				
Tab 2	50			50	
Tab 3	75			25	
Tab 4	50				50
Tab 5	50		50		
Tab 6	75		25		
Formulations of pellets					
	Bacterial suspension	Skim milk	Lactose	Microcrystalline cellulose	Inulin HP
Pel 1	30	25	40	10	25
Pel 2	30	15	40	10	35

### 2.2.2.5 Coating

Both mini-tablets and pellets (100g) were coated with Eudragit FS30D (a coating material designed to deliver drugs to the colon), to obtain a 15% weight gain. Glyceryl monostearate (glidant) was added to 70°C heated water and stirred at high speed for 20min until a fine dispersion was obtained. After cooling down to room temperature this dispersion was slowly poured into the Eudragit FS30D dispersion while gently stirred with a magnetic stirrer. The coating dispersions were passed through a 0.5 mm sieve before use. A fluid-bed coating apparatus (Strea, Niro-Aeromatic, Switzerland) was operated at 30°C, at a spray rate of 4 g/min and atomising pressure of 1.5 atm with a 1mm diameter nozzle. After coating both coated tablets and pellets were cured in the apparatus for 30 min at the same temperature as the coating process.

### 2.2.2.6 Water content assay

The water content of the dosage forms was determined gravimetrically in a moisture balance (Sartorius moisture analyzer, Goettingen, Germany). 300mg samples were dried to constant weight by infrared radiation at 105°C.

### 2.2.2.7 Viability of the microorganisms

The number of viable cells or spores was determined by the agar plate method before and after freeze-drying, tableting, pelletisation and coating, and throughout six months of storage. Samples of each dosage form were re-hydrated and serial dilutions

were plated in duplicate. The assessment of the survival rate of the microorganisms in the coated tablets or pellets was performed by submitting the coated tablets and pellets to a HCl solution (pH 1.5, at 37°C for 2 hours) and then to a 0.05M phosphate buffer solution (pH 7.4, at 37°C) (European Pharmacopoeia 5, 2.9.3 (European Pharmacopoeia).

#### **2.2.2.8 Storage conditions**

All dosage forms were kept in plastic tubes over silica gel in closed glass containers (desiccators), at 4°C, for a maximum period of 6 months.

### **2.3 Results**

#### **2.3.1 Bacterial de-hydration**

A comparison between the different processes of de-hydration of bacteria has proved the use of freeze-drying technique to obtain a higher viability when compared with other techniques (Meng et al., 2008). Initially the stabilizing capacity of two different protective media was tested. *L. plantarum* was freeze-dried (FD) in its growth medium, MRS broth, and in skim milk, both enriched with 1% of glycerol. The viable count decreased less than 1 log unit after FD and was comparable in both media. However, the MRS broth matrix was brittle and did not possess the good boosting properties of the milk. The results confirmed the reported suitability of milk as a protective matrix in the process of lyophilization of bacteria (Huyghebaert et al., 2005a, Carvalho et al., 2004).

In a different set of experiments the inclusion of glycerol and mannose in the formulation of the cakes was considered. After 2 months of storage the decrease of the bacterial viability after freeze-drying was comparable between cakes containing glycerol and mannose.

The cakes produced by freeze-drying at room temperature (25°C) for 24h showed residual moisture content of 6-7%, which decreased to 5% (48h) and to 2-3% after 72h, remaining constant afterwards. Thus, drying at room temperature was deemed convenient and considered in future cycles of drying.

Following these findings, cakes were prepared from bacteria harvested at maximum concentrations of 10<sup>9</sup>cfu/ml for *L. plantarum* and LGG and 10<sup>8</sup>cfu/ml for *L. bulgaricus*, and 10<sup>7</sup>cfu/ml for *B. subtilis* spores in skim milk and glycerol. It was found that 1ml of

a bacterial or spore suspension in skim milk with glycerol was converting into approximately 0.1g of freeze-dried powder. Figure 2.1 shows the decrease of the number of viable bacteria from the moment of preparation of the bacterial suspensions (week -1: overnight cultures) until the end of storage (week 24). Week 0 refers to the beginning of storage immediately after freeze-drying.

*L. plantarum* and LGG showed a minor decrease of bacterial counts after drying, i.e. from  $3.4 \times 10^9$  to  $1.2 \times 10^9$  cfu/ml and from  $1.7 \times 10^9$  to  $1.1 \times 10^9$  cfu/ml respectively. *L. bulgaricus* was the most sensitive strain to drying: its viability dropped from  $3.8 \times 10^8$  to  $0.5 \times 10^8$  cfu/ml. The latter, as the other strains, have shown a constant viability over the 6 months of storage. The spores of *B. subtilis* were resistant to both processing and storage without any change in the survival rate (Figure 2.1).

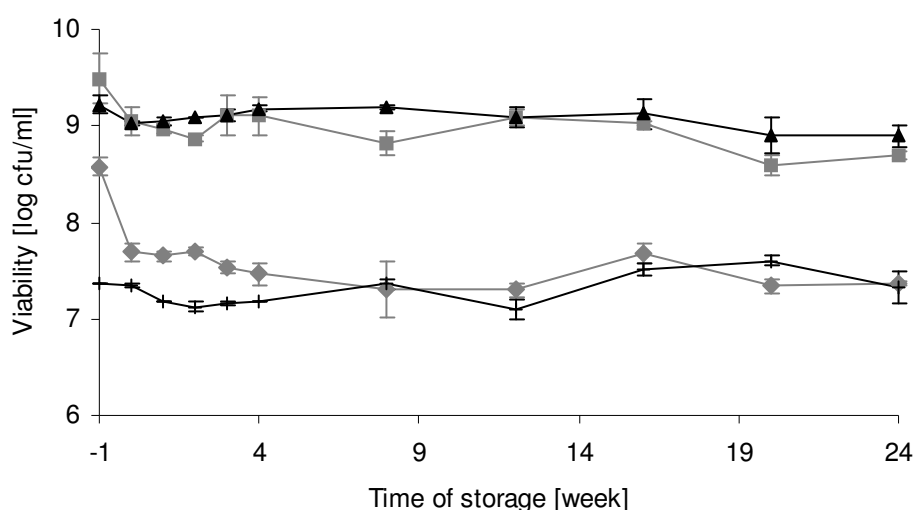


Figure 2.1 Viability of freeze-dried bacteria during storage (mean and SD, n=3). (The first point in each line represents the number of viable bacteria in each suspension before freeze-drying) (■ *L. plantarum*; ▲ LGG; ◆ *L. bulgaricus*; + *B. subtilis*).

### 2.3.2 Tableting

The freeze-dried powders were converted into mini-tablets according to the formulations considered in Table 2.1 (upper part). The production of mini-tablets was designed to allow comparison with pellets as multi unit dosage forms, and also to impose high shear forces on the materials under compression. The results in Table 2.2, for one strain only, summarize the observations made for all strains, because: a) the results for 1kN compaction force applied were not significantly different from the ones observed for a 2kN force and thus are not shown, and b) the patterns observed for *L. bulgaricus* were identical to the ones observed with the other strains.

As anticipated the tablet's height was dependent on the force applied to the materials: lower the force higher the height (Table 2.2). By filling the tablet dies manually it was possible to keep the weight of the tablets approximately constant for all batches. Even though, in some cases tablets were produced with a significant weight variation due to the poor flow ability of the powders (e.g. tab 4-6, inulin, Table 2.2) suggesting that this excipient may not be the most adequate for tableting. Table 2.2 (upper part) shows that microcrystalline cellulose had the best effect on tableting by producing tablets with higher tensile strength whereas the inclusion of inulin in the formulations did not improve the properties of the frozen cakes (Table 2.2). The densities of the tablets were not indicative of a particular formulation. Changes on density (Table 2.2) were not related to the tensile strength nor to the tableting force used failing to show a clear relationship between the porosity of the formed tablets (related to their density) and the number and intensity of bonds between particles (reflected by the tensile strength). The disintegration times of the tablets have shown that the presence of microcrystalline cellulose (tab 2 and 3, Table 2.2) in the formulation delayed the disintegration of the tablets (probably due to the formation of a matrix system) whereas tablets produced from freeze-dried cakes (tab 1, Table 2.2) did show the lowest disintegration times due to the high affinity for water of the lyophilizates. Tablets containing inulin have shown behaviour in between the previous ones due to the fact that inulin neither forms a matrix nor has affinity for water as the freeze-dried materials (tab 4-6, Table 2.2).

Table 2.2 Summary of the properties of mini-tablets and pellets produced with *L. bulgaricus* and *L. plantarum*, respectively.

	Dosage Form	Force on tableting [kN]	Thickness [mm]	Weight [mg]	Crushing force [N]	Tensile strength [Mpa]	Density [g/cm <sup>3</sup> ]	Disintegration time [min]
<i>L. bulgaricus</i>	Tab 1	2	1.00±0.03	6.04±0.42	30.5±6.0	7.77	1.502	2
		5	0.83±0.05	5.96±0.26	30.9±1.6	9.49	1.403	4
	Tab 2	2	1.04±0.04	7.06±0.77	41.0±2.9	10.04	1.609	7
		5	1.11±0.04	7.72±0.54	49.8±0.1	11.43	1.842	11
	Tab 3	2	1.04±0.03	6.41±0.49	42.9±3.7	10.51	1.901	6
		5	0.86±0.02	6.32±0.21	42.4±8.5	12.56	1.812	8
	Tab 4	2	1.12±0.05	6.85±0.92	26.3±3.2	5.98	1.890	6
		5	0.92±0.10	6.11±0.58	27.9±3.8	7.73	1.532	6
	Tab 5	2	1.14±0.07	7.19±0.45	33.8±1.5	7.55	1.531	3
		5	1.04±0.06	6.71±0.85	30.7±0.9	7.52	1.732	4
	Tab 6	2	1.11±0.05	7.04±0.42	24.8±4.6	5.69	1.683	3
		5	1.00±0.07	7.14±0.50	32.7±7.4	8.33	1.508	6
<i>L. plantarum</i> Pellets			1.18	3.50±0.23	5.0±0.2		1.462	8

A more distinctive difference between tablets was observed when the viability of each microorganism was considered. Figure 2.2 represents the log of cfu/g (colony forming unit *per* gram) of tablet mass as a function of time starting from the production of the freeze-dried bacteria (week -1), to the production of tablets (week 0) until the end of storage (week 24).

A decrease of 1 to 2 log units in the bacterial viability was observed immediately after compression for all strains (e.g.  $1.2 \times 10^{10}$  to  $7.8 \times 10^8$  cfu/g for *L. plantarum*,  $1.0 \times 10^{10}$  to  $2.5 \times 10^8$  cfu/g for LGG and  $5.0 \times 10^8$  to  $4.2 \times 10^7$  cfu/g for *L. bulgaricus*; average of results for tab 1, 4,5 and 6, Table 2.3), except of the spores ( $2.2 \times 10^8$  to  $1.6 \times 10^8$  cfu/g for *B. subtilis* spores; average of results for tab 1, 4-6, Table 2.3). Figure 2.2 (tab 1, left column) shows that the viability of the micro-organisms in the freeze-dried powders was identical to the one observed for the mixtures containing inulin (tab 4-6, Figure 2.2).

Looking at the *B. subtilis* behaviour it suggests that the spores are robust enough to support changes related to the formulation (inclusion of microcrystalline cellulose or inulin), to the force applied (1 to 5 kN) or to the proportion between the freeze-dried cake and the excipient (Figure 2.2, lower graphs). Moreover, the desiccated form remained stable for the period of observation (6 months, Figure 2.2). By contrast *L. bulgaricus* and LGG have shown major decreases of their viabilities, particularly when microcrystalline cellulose was used in the formulation. The presence of microcrystalline cellulose not only promoted a sudden decrease during compression of both micro-organisms (e.g.  $1.0 \times 10^{10}$  to  $2.3 \times 10^7$  cfu/g for LGG and  $5 \times 10^8$  to  $3 \times 10^6$  cfu/g for *L. bulgaricus*, tab 2 and tab 3), but was also unable to keep their stability, reaching a minimum by the end of the third month of storage below which detection was not possible (Figure 2.2, tab 2 and tab 3).

*L. plantarum* was the most resistant to tableting among the lactobacilli strains. Its viability decreased, when compacted alone or with inulin was less or equal to 1 log until the end of storage (Figure 2.2, upper graphics and Table 2.3) while the viability loss in the same formulations for LGG and *L. bulgaricus* reached 2 log units compared with the initial cell concentration after tableting (Figure 2.2, middle graphics and Table 2.3).

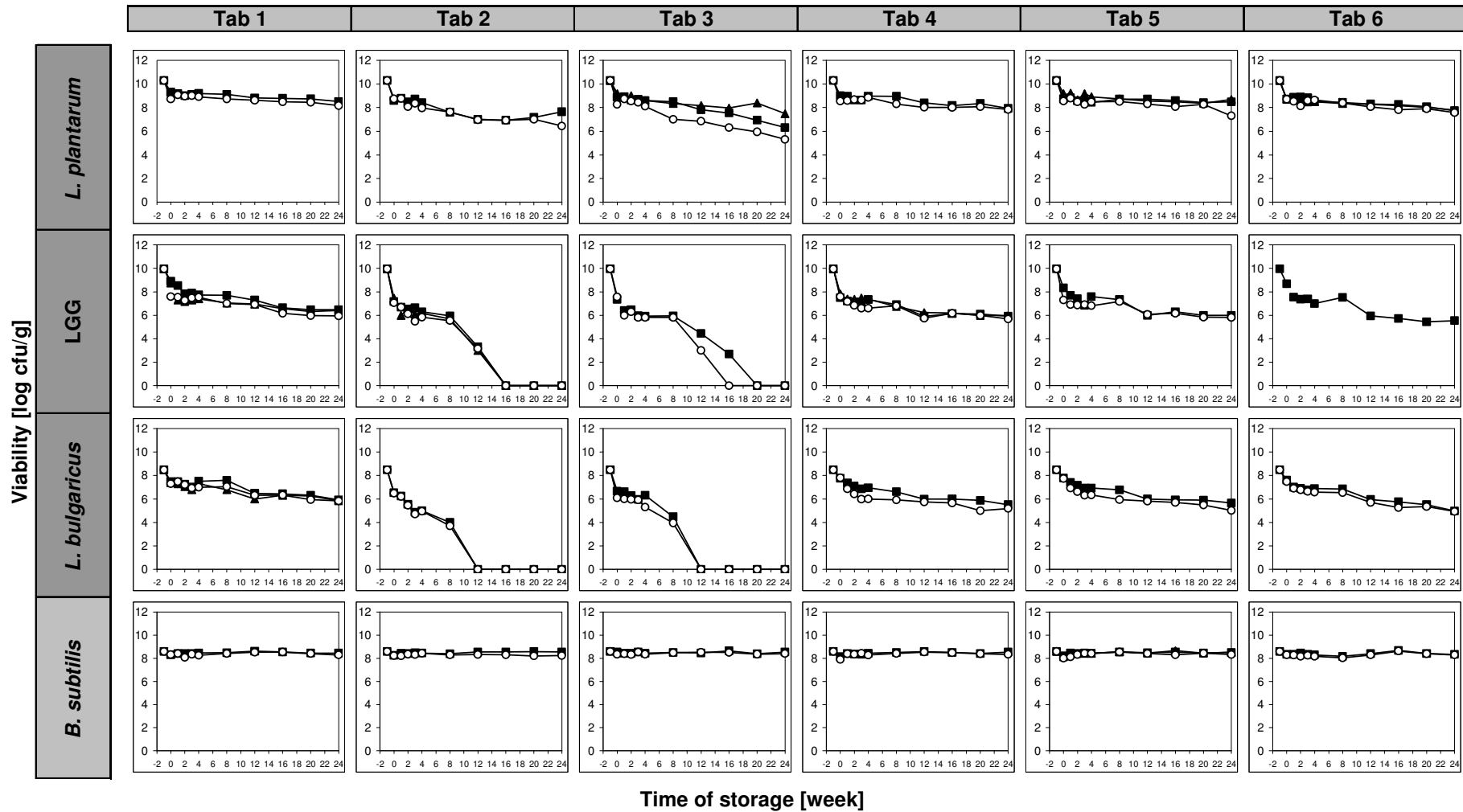


Figure 2.2 Bacterial viability in mini-tablets during storage [log cfu/g]. The initial point in each graph represents the viable count in the freeze-dried powders before tableting. (▲ 1kN; ■ 2kN; ○ 5kN)

### 2.3.3 Pelletisation

Pelletisation started by kneading the powders in the formulation with water. It was observed that a 1:1 mixture of skim milk and inulin resulted in a quickly solidifying mixture unsuitable for extrusion. Kneading of skim milk alone resulted in sticky to semi-liquid mixture whereas kneading of inulin alone gave a sticky mass originating rod pellets after extrusion and spheronisation. These limitations were overcome by inclusion of lactose and microcrystalline cellulose in the formulations (pel 1 and pel 2, Table 2.1, lower part).

Figure 2.3 shows the changes of the numbers of viable bacteria throughout the process of pelletisation. Pellets prepared according to Pel 1 formulation (Table 2.1, lower part) have shown a continuous decrease of the number of viable cells. Again, the most sensitive were the *L. bulgaricus* and LGG, resulting in a loss of viability of approximately 3 log units due to kneading, extrusion and fluid bed drying, but not spheronisation (Figure 2.3). The viability kept decreasing throughout storage ( $4.5 \times 10^6$  cfu/g to undetectable levels in four months for LGG and  $1.8 \times 10^5$  cfu/g to undetectable levels in one month for *L. bulgaricus*, 'Pellets', Table 2.3 and Figure 2.4). In the present study *L. plantarum* and, particularly *B. subtilis* were stable throughout the process of extrusion-spheronisation and subsequent drying (Figure 2.3) and in storage ( $4.1 \times 10^9$  and  $1.7 \times 10^9$ , *L. plantarum*,  $8.0 \times 10^6$  and  $13 \times 10^6$ , *B. subtilis*, Table 2.3). As an attempt to confirm the positive effect of inulin on the bacterial viability, pellets with higher amount of inulin were considered (pel 2, Table 2.1, lower part). Increasing the content of inulin, with a concomitant decrease on skim milk content led to a destabilizing effect during storage, i.e., a 1 log unit decrease (at the end of the third month of storage), while the viable count in the first formulation remained constant during storage. This effect has also been observed in storage of pellets obtained by layering of *L. lactis* dispersions in skim milk and inulin over inert pellets (Huyghebaert et al., 2005c).

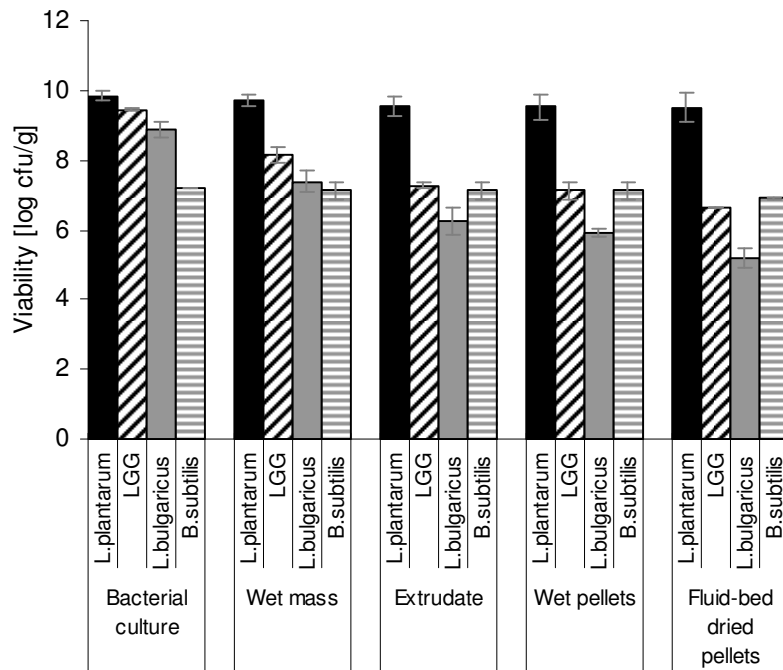


Figure 2.3 Bacterial viability during pelletisation (mean and SD, n=3).

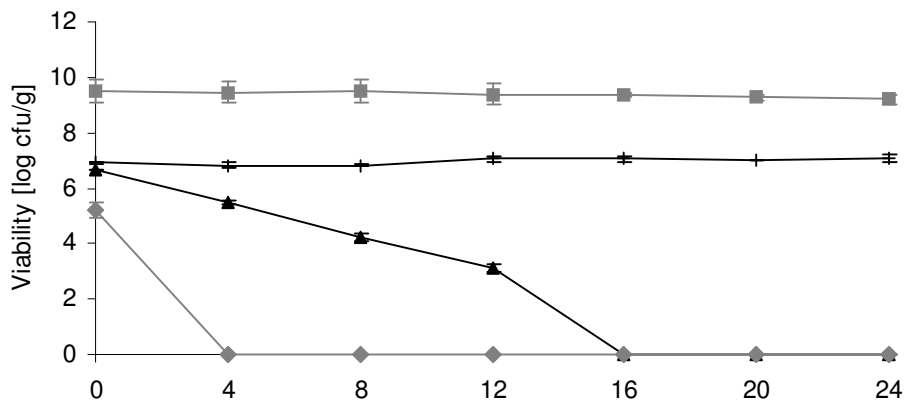


Figure 2.4 Bacterial viability in non-coated pellets during storage (mean and SD, n=3). (■ *L. plantarum*; ▲ LGG; ◆ *L. bulgaricus*; + *B. subtilis*)

### 2.3.4 Coating

Regarding the application of the coat for colonic delivery, a proper protection was reached when 15% coat (by weight gain) was applied. For this amount of coating material applied both tablets and pellets remained intact in acidic medium for 2 hours (HCl, 0.1N at 37°C) with bacterial viability preserved in comparison to the uncoated cores. When the



tablets and pellets were placed in phosphate buffer solution (pH 7.4, 37°C) they have disintegrated after 1.5 hours.

Coating of both tablets and pellets with Eudragit FS30D caused an initial viability decrease, less than 1 log unit, when compared to the cores ('Coated' Table 2.3 and Figure 2.5 a,b). It is interesting to point out that the viability during storage was not affected by coating, i.e. the viability profiles of coated tablets and pellets decreased as before of the uncoated tablets and pellets ('Coated' Table 2.3 and Figure 2.5 a,b).

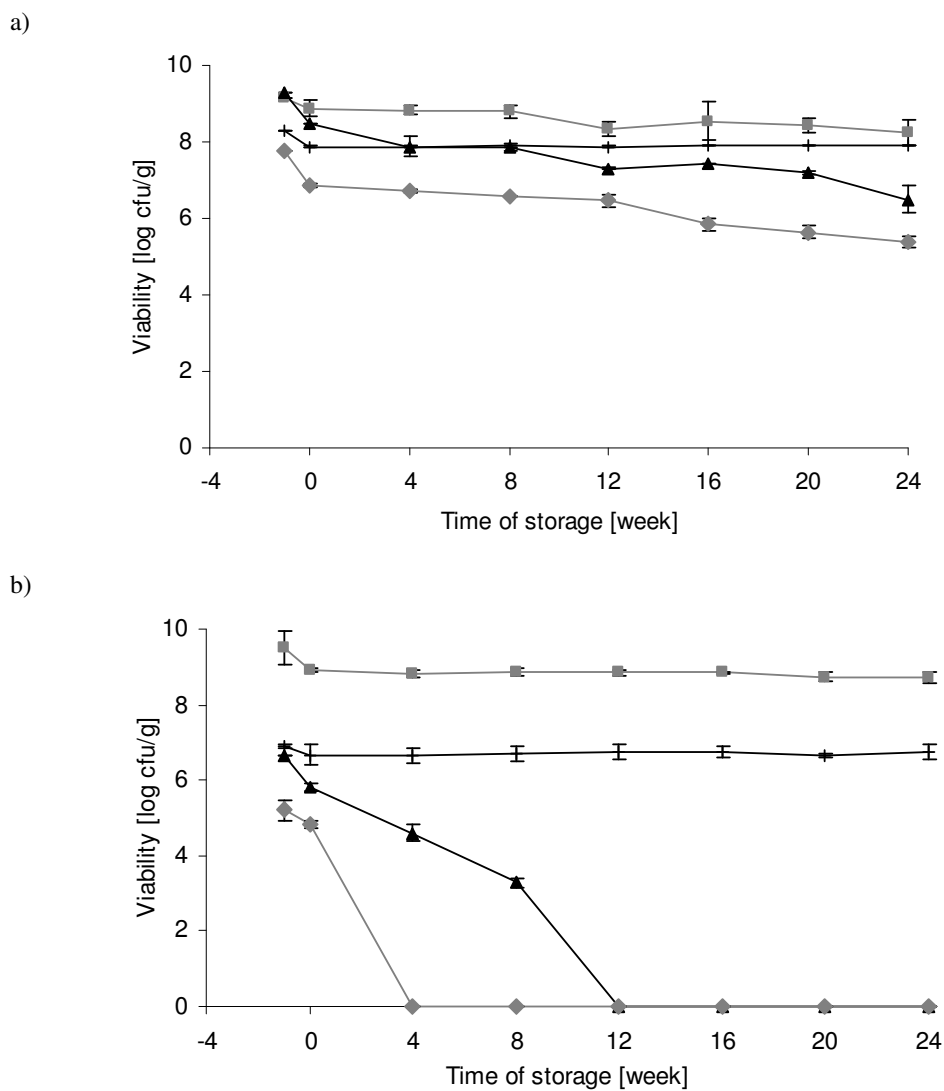


Figure 2.5 Bacterial viability before and after coating and throughout storage (mean and SD, n=3) in a) mini-tablets (the first point in each line represents the viable count in the tablets before coating) and b) pellets (the initial point in each graph represent the viable count in the pellets before coating).

(■ *L. plantarum*; ▲ LGG; ◆ *L. bulgaricus*; + *B. subtilis*)

## 2.4 Discussion

Previous tests have shown that processing of bacterial suspensions in the presence of components such as skim milk and glycerol ended up in high survival rates of bacteria in the resulting product (Huyghebaert et al., 2005a; Carvalho et al., 2004). Therefore, it was deemed convenient to incorporate one or more of these materials in the formulations at early stages of production. These materials by protecting (throughout processing) and nourishing the bacteria (upon release from the dosage form) promote high viability rates after processing. Inulin adds other advantages to the product, namely by reducing the risk of colonic cancer to the patient (Verghese et al., 2002, Roberfroid, 2000) and by providing enhanced filler and binding properties for direct compression of materials (Eissens et al., 2002).

Table 2.3 Viability of the examined strains in the different pharmaceutical formulations (1)

Strains	Storage at 4°C	Lyophilizates	Mini-tablets	Pellets	Coated mini-tablets (2)	Coated pellets (2)
	[month]	[cfu/g]	[cfu/g]	[cfu/g]	[cfu/1,15g]	[cfu/1,15g]
<i>L. plantarum</i>	0	1,15E+10	7,82E+08	4,08E+09	7,50E+08	8,30E+08
	3	1,30E+10	3,01E+08	2,90E+09	3,00E+08	7,10E+08
	6	5,00E+09	1,60E+08	1,70E+09	2,30E+08	5,25E+08
LGG	0	1,06E+10	2,53E+08	4,50E+06	3,50E+08	6,80E+05
	3	1,30E+10	4,86E+06	1,30E+03	2,00E+07	0,00E+00
	6	8,00E+09	1,20E+06	0,00E+00	3,45E+06	0,00E+00
<i>L. bulgaricus</i>	0	5,00E+08	4,22E+07	1,75E+05	7,00E+06	6,50E+04
	3	3,50E+08	1,17E+06	0,00E+00	2,95E+06	0,00E+00
	6	2,30E+08	3,78E+05	0,00E+00	2,50E+05	0,00E+00
<i>B. subtilis</i>	0	2,20E+08	1,62E+08	8,00E+06	7,60E+07	5,00E+06
	3	1,45E+08	3,07E+08	1,20E+07	7,40E+07	5,75E+06
	6	2,20E+08	2,59E+08	1,25E+07	8,10E+07	5,75E+06

(1) The represented values of viability are average values from the viabilities of the formulations of lyophilizates compacted alone or with inulin ST/HP.

(2) 1.15g of coated material equals 1g of non-coated material.

The fact that the cell numbers after drying remained in the same order of magnitude as in the overnight cultures suggests that on the one hand, adequate processing conditions were reached and, on the other hand, the viability of bacteria was more affected by changes in the water content present in the environment and particularly in the dehydration of the cells. Furthermore, the drying process is a key to high survival rates: FD allowed a higher survival than air drying, as observed throughout extrusion, for instance.

The consequent compaction of the freeze-dried bacteria showed higher survival rates of *L. plantarum* during compression and storage compared to LGG and *L. bulgaricus*. Moreover, *L. plantarum* demonstrated a higher survivability than the other lactobacilli when compacted with microcrystalline cellulose, which was unable to protect the bacterial cells from the forces exerted over them in the process of tablet formation and from the environment throughout storage. It is clear that bacterial cells differ in their susceptibility according to strains, species, growth conditions, suspending medium, freeze-drying and tableting conditions. The genus *Lactobacillus* comprises more than 100 species (Felis and Dellaglio, 2007) widely differing in biochemical, ecological and molecular-biological properties. This large variation is often reflected by the wide range of G+C content of DNA from 32 to 53 mol%, a span twice as large as usually accepted for a single genus, and by the lack of significant DNA/DNA homology between most of the species. *L. plantarum*, followed by LGG, exhibits a wide range of fermented carbohydrates, while *L. bulgaricus* ferments only a few carbohydrates, i.e. fructose, lactose and glucose. *L. plantarum* has been isolated from dairy products and environments, silage, sauerkraut, pickled vegetables, sour dough, cow dung and human mouth, intestinal tract and stools, and from sewage (Kandler and Weiss, 1986). LGG has been isolated from human intestinal tract (Gorbach et al., 1987) and *L. bulgaricus* is adapted to specialized niches, i.e. sour milk and cheese (Kandler and Weiss, 1986). Thus, it is not surprising that from all described characteristics of the three *Lactobacillus* spp. one can recognize that *L. plantarum* is less fastidious and more adaptive to different conditions than LGG and *L. bulgaricus*. These properties of *L. plantarum* could be the reason for its higher resistance to the different processing conditions (e.g. freezing, drying, compression forces and excipients). However, it is important to understand the mechanism of cellular injury, in order to recognise the effect of the different processes and excipients on the loss of viability. Morphological, biochemical and genetic studies are required to show possible changes brought by the variable conditions.

The resistance of *B. subtilis* to freeze-drying and tableting may be explained by the fact that the spores wall and structure could support shear, tension or compression forces generated in the process of compaction. The strain dependent stability in freeze-drying and tableting were also discussed in several reports (Maggi et al., 2000; Stadler and

Viernstein, 2003). Some authors (Huyghebaert et al., 2005a) observed a drop of nearly 1 log unit in the survival rate of the bioengineered *L. lactis* after its compaction (52MPa) into mini-tablets. In one week of storage a further 25% decrease in the viability at low relative humidity (RH) and a complete viability loss of viability at 60% RH was observed. However, the effect of pressure over bacteria is contradictory. Different conclusions were reached by other authors (Stadler and Viernstein, 2003) who did not observe a reduction of *L. acidophilus* ( $10^9$  cfu/tablet) during six months of storage at 10 or 20°C. These authors considered a pressure of 25.5 to 63.7MPa. In a different study (Maggi et al., 2000) the authors described the different sensitivities of ten *Lactobacillus* strains to different excipients and processing conditions and observed a stable viability throughout one year. In both studies (Maggi et al., 2000, Stadler and Viernstein, 2003) the higher stability reported could be due not only to the individual strain characteristics but also to the dosage form. Both research groups prepared bigger tablets (10mm) than the ones discussed in the present work and, certainly the forces (namely shear forces) over individual particles or cells observed in tableting are different and, consequently, differences should be expected regarding the performance of the bacteria.

As anticipated an increase of the compaction force was generally reflected by an increase of the tensile strength, suggesting that the particulate powders deformed plastically under pressure. Direct compression of the freeze-dried cakes required the use of microcrystalline cellulose or inulin to overcome some technological problems of the cakes namely stickiness, poor flow or poor compaction properties. The presence of plastic materials (e.g., microcrystalline cellulose) is expected to affect the viability of bacteria because, by definition, these materials under pressure deform in all directions. On the other hand, materials that have some degree of brittleness, such as inulin (Eissens et al., 2002) can provide some degree of protection by accommodating some stresses due to compression forces. This may explain the poor protection provided by the microcrystalline cellulose on protecting the bacterial cells from the forces exerted over them in the process of tablet formation and from the environment throughout storage.

Pelletisation recurs traditionally to lactose and microcrystalline cellulose (Fielden et al., 1993). However, the above discussion has shown that microcrystalline cellulose has a deleterious effect on the stability of the microorganisms, thus it had to be kept at lower

levels. In fact extrusion also requires the application of large forces on the materials. On the other hand, inulin (HP) and skim milk were included in the formulations (Table 2.1) providing some cushioning effect to the cells under stress together with the prebiotic activity. The technology of extrusion and spheronisation is complex and a good product is obtained when a combination of several factors is reached, namely plasticity of the wet masses, amount of granulation fluid and proper parameterisation of both extruder and spheroniser (Fielden et al., 1993). Due to the high pressure applied to the material, shear and frictional forces are present in the process. Furthermore the process is very sensitive to the amount of water present in the wet mass. In this work the amount of water is also of paramount importance for the preservation of bacterial viability, as discussed before. The observed viability decline could be due to the combined effect of the forces applied (also present in tableting) with the continuous decrease of the water content observed in the production of the pellets, which could have subjected the bacterial cells to osmotic shock (Mille et al., 2004), the severity of which determined the bacterial viability. Some authors (Huyghebaert et al., 2005a) reported a high sensitivity of *L. lactis* to pelletisation: they observed a 2 log units decrease in the survival rate of *L. lactis* due to extrusion-spheronisation and fluid bed drying processes, 2 log units more in 1 week storage at 10% RH and complete viability loss in 1 week at 60% RH.

Probiotic bacteria are commonly required to exert their action in the far small intestine or in the colon. Coating of active cores is an often-used strategy to reach this aim. Coating was observed to be substantially less deleterious relative to the viability of microorganisms compared to tablets and pellets. Taking into consideration the viability of LGG and *L. bulgaricus*, the small reduction of the viability can be explained by a series of reasons. The cores (both pellets and mini-tablets) showed a low water content, i.e., they were coated as dry cores and thus, no change on the water content was observed, except at their surface, where the application of the coating suspension did not promote the presence of high amounts of water at the surface of the cores, thus, the latter were not wetted. The initial viability decrease during coating was probably due to the loss of viability of cells present on the surface of the pellets and mini-tablets. Possible interference of bacteria with Eudragit FS30D affecting their viability cannot be ruled out. Furthermore the coat promoted a barrier to the environment. Although the coat was not

completely impermeable it probably prevented the oxygen (and moisture) to enter into contact with the bacteria, thus anaerobic conditions were promoted. Consequently, it was not surprising that further significant decrease of viable bacteria was not observed due to coating.

## 2.5 Conclusions

The work has identified the possibility of producing solid dosage forms containing viable bacteria as active moieties. The dosage forms considered reflect the most important ones administered by oral route. The work has also shown how, by different formulation and processing designs (powder, pellets, tablets and coated pellets and tablets), one can promote the release of bacteria in different segments of the digestive tract (stomach, small intestine or colon) according to the desired objective and function.

The different bacteria chosen (3 vegetative forms and 1 spore form of prebiotic) have shown different viabilities to formulation and processing with decreasing viability as follows: *B. subtilis* (spores), *L. plantarum*, LGG and *L. bulgaricus* (vegetative forms).

The most important factor to be considered in processing bacteria is the hydration state of the cells. The process of de-hydration is critical for obtaining a high survival rate. Freeze-drying has allowed high viability (less than 1 order of magnitude decrease) whereas drying observed throughout the process of extrusion and spheronisation has a high effect on the viability of bacteria. Also important is the fact that nutrients (skim milk, glycerol or mannose) and cryoprotectors should be present at early stages of processing (before de-hydration) to promote the stabilisation of bacteria.

Tabletting was possible once compression aids (e.g. microcrystalline cellulose) were present to overcome the flow ability, stickiness and compaction problems of the freeze-dried powders. However, microcrystalline cellulose presented a deleterious effect on the viability of bacteria. Here the compaction force seems to affect little the viability of the bacteria, particularly when direct compression of the materials was possible, due to the absence of changes on the residual water content of the materials.

Pellets with intended characteristics were obtained from mixtures containing microcrystalline cellulose and lactose. The fact that bacteria were present in a suspension

(not in the dry state) and the process of extrusion and spheronisation promotes a continuous and slow drying of materials, both affected the viability of the bacteria throughout the process.

Finally coating of either mini-tablets or pellets did not show major problems. The viability of the bacteria did not decrease further due to coating.

To assure a high viability after storage both the temperature and relative humidity must be kept low.

## **CHAPTER 3**

### **PRESERVATION OF THE VIABILITY AND ANTIBACTERIAL ACTIVITY OF *LACTOBACILLUS* SPP. IN CALCIUM ALGINATE BEADS**



### **3.1 Introduction**

Recently, the problems associated to the spread of antibiotic resistances among bacteria has led to a new interest in bacteriotherapy, a practice that makes use of commensal bacteria or beneficial bacteria to prevent or treat colonization of the host by pathogens (Peral et al., 2009; Huang et al., 2009; Skovbjerg et al., 2009). This approach is based on the competitive exclusion principle where the harmless commensal or probiotic bacteria (e.g. lactobacilli) successfully compete with potential pathogens for the same site (Huovinen, 2001; Reid et al., 2001) (see 1.1.2).

However, to produce therapeutic benefits, sufficient number of viable and biologically active lactobacilli must be present. Consequently, finding optimal technological conditions to keep the organisms alive and active during processing and storage is a critical problem. Immobilization technology (e.g. entrapment of lactobacilli in alginate matrix) can be used as approach for stabilization, aiming to retain high density of active lactobacilli cells and protect them from hostile environments (see 1.2.7).

### **3.2 Materials and Methods**

#### **3.2.1 Materials**

Glycerol, sodium chloride and calcium chloride (Sigma-Aldrich, Chemie, Germany), disodium hydrogen phosphate and potassium di-hydrogen phosphate (Pancreac, Barcelona, Spain), ethylenediaminetetraacetic acid (EDTA, Merck, Darmstadt, Germany), sodium alginate (Protanal LF 10/60, FMC BioPolymer, Drammen, Norway) and skim milk (Difco, Detroit, USA) were used. The bacterial growth media used were de Man-Rogosa-Sharpe agar and broth (MRS), Mueller-Hinton (MH), Slanetz agar, Cetrimide agar and Drigalski lactose agar (Biokar Diagnostics, France) and CM 0085 mannitol salt agar (Chapman), (Oxoid Ltd., England).

## 3.2.2 Methods

### 3.2.2.1 Bacterial strains and culture conditions

The lactobacilli strains examined, *Lactobacillus plantarum* ATCC 8014, *Lactobacillus rhamnosus* strain GG ATCC 53103 (LGG), *Lactobacillus delbrueckii* subsp. *bulgaricus* CIP 101027 and *Lactobacillus delbrueckii* subsp. *lactis* CIP 53.61, were grown overnight at 37°C on MRS agar and in broth.

The multi-resistant clinical isolates were cultivated overnight at 37°C on selective agar media: a methicillin-resistant *Staphylococcus aureus* FFUL 29593 (Gaspar-Marques et al., 2006) was grown on Chapman; a vancomycine-resistant *Enterococcus faecalis* FFUL H164 (Gaspar-Marques et al., 2006) on Slanetz; a VIM-2-metallo-β-lactamase-producing *Pseudomonas aeruginosa* FFUL 1396, on Cefrimide and CTX-M-15-β-lactamase producing strains: *Escherichia coli* FFUL 125269 (Brizio et al., 2006) and *Klebsiella pneumoniae* FFUL 193K (Conceição et al., 2005) on Drigalski.

All stock cultures were maintained at -80°C in growth medium supplemented with 15% glycerol, except *L. lactis*, which was supplemented with 50% glycerol.

### 3.2.2.2 Preparation of bacterial inocula

The lactobacilli were used as whole cultures, culture filtrates and washed cells, as follows: Preparation of whole cultures - Whole cultures were the overnight broth cultures produced at 37°C; Preparation of culture filtrates - Culture filtrates were obtained by centrifugation of the whole cultures (at 4000rpm for 20min at 4°C) and filtration of the supernatants through cellulose acetate membrane filters (pore size 0.2µm); Preparation of washed cells - The cells harvested by centrifugation of the whole cultures were washed twice and resuspended to initial volume in physiological saline.

An inoculum of each multi-resistant strain was prepared by suspending single colonies, from the overnight agar cultures, in physiological saline to result in an optical density equal to 0,5 McFarland that corresponded to approximately  $1 \times 10^8$  cfu/ml (colony forming units per ml).

### 3.2.2.3 Co-culture experiments

Well diffusion method: The antibacterial activity spectra of the lactobacilli strains were determined by well diffusion method using the five multi-resistant clinical isolates as indicator strains. The assay was performed by using lactobacilli whole cultures, culture filtrates and washed cells. Aliquots (60µl) of each *Lactobacillus* inoculum were placed in wells (6-mm diameter) punched in cooled agar plates, which were previously inoculated with the multi-resistant strains by sterile cotton swabs. Two types of agar media were considered. Mueller-Hinton (MH) was used as the medium of choice for susceptibility testing of commonly isolated, rapidly growing aerobic or facultative organisms (NCCLS, 2003). However, lactobacilli are extremely fastidious organisms, requiring not only carbohydrates as energy and carbon source, but also nucleotides, aminoacids and vitamins (Kandler and Weiss, 1986). Therefore, MRS, being a specific lactobacilli growth medium, was also considered in the assay to test its effect on lactobacilli antibacterial activity. The plates were incubated overnight at 37°C. The sizes of the inhibition zones (mm) were measured.

Microdilution broth method: A microdilution broth method was carried out to study the relationship between the antibacterial activity and viability of lactobacilli cultures. The test was performed in 96-well microplates, as follows: Tenfold serial dilutions of the lactobacilli overnight cultures were prepared in physiological saline and in MRS broth (from  $10^9$  to  $10^2$ cfu/ml). 90µl of each dilution were added to the wells containing 100µl of MH broth. When physiological saline was used in the serial dilutions, the MH broth in the wells was double concentrated in order to obtain its standard concentration after the addition of the lactobacilli and multi-resistant strains suspensions (NCCLS, 2003). The inocula of the multi-resistant strains were diluted 1/10 in physiological saline, so that the addition of 10µl to the wells would result in a concentration of  $5 \times 10^5$ cfu/ml. Positive control wells contained only the microdilution media and inoculum of each multi-resistant strain. The microplates were incubated overnight at 37°C. A droplet of each overnight culture from the microdilution wells was transferred to the selective agar media of the multi-resistant strains. The plates were also incubated overnight at 37°C. The antibacterial activity of lactobacilli was determined as the lowest cell concentration of a *Lactobacillus* strain that completely inhibited the growth of a multi-resistant strain.

### **3.2.2.4 Preparation and characterisation of lactobacilli loaded calcium alginate beads**

#### **3.2.2.4.1 Preparation of lactobacilli loaded calcium alginate beads**

Four types of lactobacilli loaded alginate beads were compared: 1) beads containing entrapped, 2) entrapped and surface, 3) surface and 4) concentrated entrapped and surface lactobacilli cultures.

Beads containing entrapped lactobacilli (E beads): The encapsulation of lactobacilli overnight cultures in calcium alginate was a modification of the method described by Klinkenberg et al., 2001. Briefly, sodium alginate (1g) was autoclaved and dissolved in the overnight cultures (50ml) of each *Lactobacillus* strain by stirring at 50 rpm. Each cell-alginate mixture was then added dropwise into a sterile solution of sodium chloride (0.2M) and calcium chloride (0.05M) through pipette tips with an inner diameter of 0.5mm. Sodium chloride was used in the gelling solution to ensure a homogeneous polysaccharide concentration throughout the beads. To obtain complete gelling, the resulting 3.5mm beads were stirred at least 40 min at 50 rpm in this suspension prior to separation from the system by filtration under vacuum. The entire immobilization procedure was performed at room temperature.

Beads containing entrapped and surface lactobacilli (ES beads): Lactobacilli loaded calcium alginate beads (50g) obtained as described above, were incubated in MRS broth at 37°C for proliferation of the lactobacilli to the beads surfaces (Prevost et al., 1985). Incubation of the beads was ceased in 4.0h (*L. plantarum*), 4.5h (LGG) and 6.0h (*L. bulgaricus* and *L. lactis*) when the concentration of free lactobacilli cells corresponded to  $10^7$  cfu/ml for *L. plantarum*, LGG and *L. lactis* and  $10^6$  cfu/ml for *L. bulgaricus*.

Beads containing surface lactobacilli (S beads): Blank alginate beads, containing no lactobacilli but only MRS broth, were incubated for 16h at 37°C in MRS broth inoculated with 100µl of *L. plantarum* overnight culture.

Beads containing concentrated entrapped and surface lactobacilli cell suspensions (C<sub>ES</sub> beads): Two different approaches, 1) centrifugation and 2) repeated fermentations, were compared in concentrating *L. plantarum* suspensions immobilized in alginate beads.

1) *L. plantarum* overnight cultures were centrifuged (4000 rpm for 30min at 4°C) and the cells were resuspended in tenfold smaller volume of the supernatant.

2) The effect of two types of media, MRS broth supplemented with 1% CaCl<sub>2</sub> and reconstituted skim milk (10%), on concentrating immobilized *L. plantarum* cultures was studied. *L. plantarum* alginate beads (50g) were three times subsequently incubated in either medium for 4h at 37°C. Afterwards *L. plantarum* beads fermented in MRS broth with CaCl<sub>2</sub> were incubated in MRS broth for 4h at 37°C, while the beads fermented in skim milk were incubated for halftime at the same conditions.

Blank calcium alginate beads (B<sub>C</sub>), containing only MRS broth, were used as controls.

All types of beads were frozen at -80°C for 24h and dried under vacuum at room temperature, 0.035mbar for 24h (Christ Alpha 1-4, B. Braun Biotech International, Germany). The beads produced were kept over silica gel, at 4°C, for a period of 6 months. These beads were considered for further characterisation. Scanning electron microscopy, determination of calcium content, physical properties and release of lactobacilli from beads were performed only with *L. plantarum* loaded beads because S- and C<sub>ES</sub>-beads were prepared only with *L. plantarum*.

#### **3.2.2.4.2 Scanning electron microscopy**

Samples from the beads were mounted on metal stubs with double sided adhesive tape, coated with gold using fine coater (JEOL JSM-1200, Japan) under vacuum and 15mA at room temperature. The morphology of coated samples was examined by scanning electron microscopy (SEM; JEOL JSM-5200 LV, Japan).

#### **3.2.2.4.3 Calcium content**

The calcium content in the prepared beads was measured by atomic absorption analysis (Perkin Elmer AAnalyst – 700). A series of standard calcium solutions (0; 1; 2; 4; 6; 8 and 10mg/l) was prepared from: 0, 0.5, 1, 2, 3, 4 and 5ml calcium stock solution

(100mg/l), 10ml EDTA solution (37.7g/l) and deionised water up to 50ml. Samples (100mg) from beads were dissolved in sodium citrate solution (1%, pH 6.0) and then diluted in EDTA solution (7.5g/l) and deionised water. The calcium content in the samples was determined by comparison with the standard solutions in the calibration curve.

#### **3.2.2.4.4 Physical properties**

The major and minor axes of beads were measured using an upright microscope Olympus CX40, Japan, equipped with an Olympus Colour View IIIu Camera and Cell D 2006 Olympus Software. Bead shape was characterized by aspect ratio, defined as the ratio between the major and the minor bead axis. The closer the values of the aspect ratio were to 1, the more spherical the bead was.

The force required to crush the calcium alginate beads was measured. The beads were placed between two horizontal plates, perpendicular to their movement and crushed (CT5, Engineering System, Nottm, UK).

#### **3.2.2.4.5 Release of lactobacilli**

Beads (100mg) were placed into tubes containing 10ml of phosphate buffered saline (PBS) (pH 7.4) and then placed into a shaking water bath at 37°C (20 osc/min). PBS was prepared by mixing of 1 ml of phosphate buffer (pH 7.5; 0.33M) with 29ml of sodium chloride (9g/l). At specified time periods (10, 30, 60, 120, 270, 360 and 600 minutes), 100µl from every test tube were withdrawn and replaced with fresh PBS. Serial dilutions were made in sterile water and plated in duplicate on MRS agar to allow counting of lactobacilli released. The percentage of bacteria released was calculated from the initial viable count of lactobacilli per gram of beads.

#### **3.2.2.4.6 Viability of lactobacilli**

The lactobacilli viability was determined before and after immobilisation and freeze-drying and during six months of storage (4°C over silica gel) by the agar plate method.

Calcium alginate was dissolved in sterile sodium citrate solution (1%, pH 6.0) followed by serial dilutions made in sterile water and plated in duplicate on MRS agar.

#### **3.2.2.4.7 Antibacterial activity**

Well diffusion method: The antibacterial activity of the immobilized into beads lactobacilli cultures was initially tested by the well diffusion method as described above. Instead of lactobacilli inocula, alginate beads containing  $10^7$  to  $10^8$  entrapped lactobacilli cells were used. Macrodilution broth method: The applied macrodilution broth method reproduced the conditions of the performed microdilution broth test. It was used to test the antibacterial activity of lactobacilli after processing into beads and during 6 months of storage.

### **3.3 Results**

#### **3.3.1 Antibacterial activity of lactobacilli before processing**

##### **3.3.1.1 Well diffusion method**

Well diffusion method was applied in a comparative study of the antibacterial activity of the lactobacilli whole cultures, culture filtrates and washed cells against the multi-resistant clinical isolates (Table 3.1). The antibacterial activity of lactobacilli against *S. aureus* and *E. faecalis* was compared on both MH and MRS agar, while against *P. aeruginosa*, *E. coli* and *K. pneumoniae* on MH agar only, as the latter microorganisms did not grow on MRS agar.

When whole cultures were used, all lactobacilli strains showed antibacterial activity against all Gram-positive and Gram-negative multi-resistant isolates, while the culture filtrates of lactobacilli were only active against the Gram-negative strains and *S. aureus* on MH agar. Although all lactobacilli strains, except *L. bulgaricus*, were able to grow on MH agar, the activity of *L. plantarum*, LGG and *L. bulgaricus* washed cells was observed only on MRS agar. Only *L. lactis* washed cells inhibited *S. aureus* on both media (Table 3.1). The sizes of the inhibition zones on MH agar were ranging from 8 to 16mm, while

on MRS from 13 to 30mm. *L. bulgaricus* produced the smallest inhibition zones on MH agar (from 8 to 10mm).

The antibacterial activity of lactobacilli against *S. aureus* was observed when the whole cultures were applied on both MRS and MH agar and the washed cells on MRS agar. The culture filtrates were active only on MH agar. However, for *E. faecalis* the lactobacilli activity was only observed when the whole cultures or washed cells were used on MRS agar, by contrast to the non-active culture filtrates. Furthermore, similar inhibition zones were caused by either whole cultures or washed cells (mean values of 17 and 16mm, respectively). Both whole cultures and culture filtrates of lactobacilli inhibited *P. aeruginosa*, *E. coli* and *K. pneumoniae*, producing similar inhibition zones (mean value of 12mm).

All further experiments were performed with whole lactobacilli cultures only, as they have shown the widest antibacterial spectrum.

Table 3.1 Antibacterial activity of the whole cultures, culture filtrates and washed cells of the lactobacilli strains represented by the size of the inhibition zones (mm).

Whole cultures	<i>S. aureus</i>		<i>E. faecalis</i>		<i>P. aeruginosa</i>	<i>E. coli</i>	<i>K. pneumoniae</i>
	MRS	MH	MRS	MH	MH	MH	MH
<i>L. plantarum</i>	22	12	18	ni	12	12	12
LGG	22	11	18	ni	12	12	11
<i>L. bulgaricus</i>	20	8	14	ni	10	10	9
<i>L. lactis</i>	28	16	18	ni	13	14	12

Culture filtrates	<i>S. aureus</i>		<i>E. faecalis</i>		<i>P. aeruginosa</i>	<i>E. coli</i>	<i>K. pneumoniae</i>
	MRS	MH	MRS	MH	MH	MH	MH
<i>L. plantarum</i>	ni	10	ni	ni	13	14	10
LGG	ni	11	ni	ni	12	13	10
<i>L. bulgaricus</i>	ni	ni	ni	ni	9	10	ni
<i>L. lactis</i>	ni	9	ni	ni	13	14	9

Washed cells	<i>S. aureus</i>		<i>E. faecalis</i>		<i>P. aeruginosa</i>	<i>E. coli</i>	<i>K. pneumoniae</i>
	MRS	MH	MRS	MH	MH	MH	MH
<i>L. plantarum</i>	14	ni	18	ni	ni	ni	ni
LGG	14	ni	18	ni	ni	ni	ni
<i>L. bulgaricus</i>	14	ni	13	ni	ni	ni	ni
<i>L. lactis</i>	30	14	14	ni	ni	ni	ni

ni = no inhibition; MRS = de Man-Rogosa-Sharpe agar; MH = Mueller-Hinton agar

### 3.3.1.2 Microdilution broth method

The microdilution broth method was used to represent the antibacterial activity of lactobacilli cultures as a function of lactobacilli viability (Figure 3.1). When the medium



in the wells comprised MH, appropriate medium for the growth of the multi-resistant strains, the lactobacilli were active at high cell densities, *i.e.*  $10^7$  to  $10^8$  cfu/ml. When MRS was added to the microdilution medium in order to facilitate the development of lactobacilli, the number of lactobacilli necessary to inhibit the multi-resistant strains was lower by 1 to 2 log units ( $10^5$ - $10^7$  cfu/ml) against *S. aureus*, *E. faecalis*, *E. coli* and *K. pneumoniae* and by 4 log units ( $10^3$  cfu/ml) against *P. aeruginosa* (Figure 3.1). Positive controls showed that both microdilution media (MH and MH+MRS) yielded satisfactory growth of the multi-resistant strains ( $10^8$  to  $10^9$  cfu/ml).

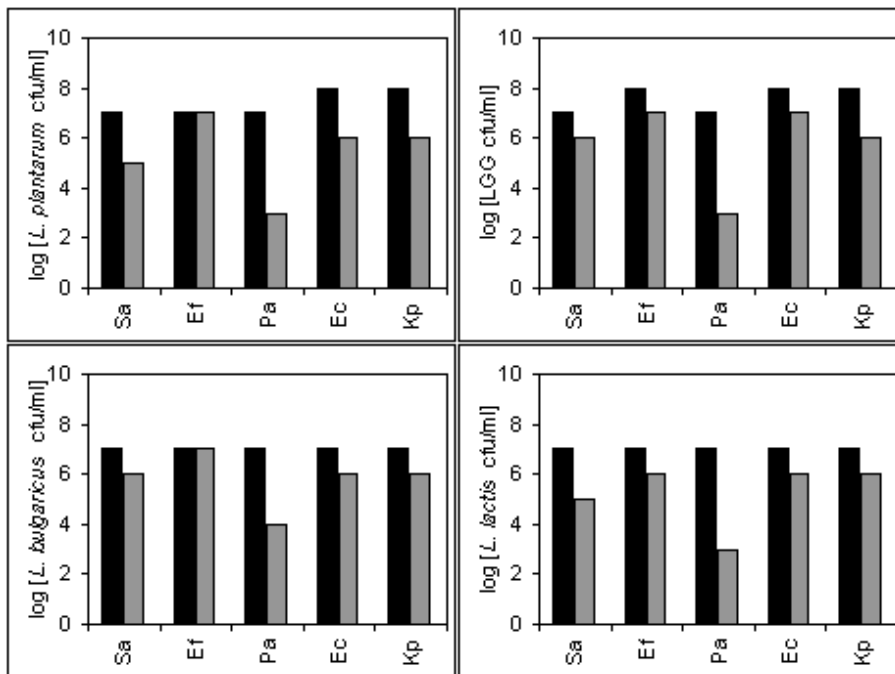


Figure 3.1 Antibacterial activity of lactobacilli determined by microdilution broth method in (■): MH+physiological saline and (▒): MH+MRS against *S. aureus* (Sa), *E. faecalis* (Ef), *P. aeruginosa* (Pa), *E. coli* (Ec) and *K. pneumoniae* (Kp), represented as the logarithm of the minimal number of lactobacilli cells necessary to inhibit completely the growth of the multi-resistant strains.

### 3.3.2 Lactobacilli loaded calcium alginate beads

#### 3.3.2.1 Physical characteristics

According to the physical characteristics two groups of beads could be distinguished (Table 3.2). The first group, represented by B<sub>C</sub> and E beads had a spindle shape, resembling prolate ellipsoids. They were characterized by smaller weight, higher calcium

content and higher force at crush. The second group, represented by ES and C<sub>ES</sub> beads, resembled more spherical ellipsoids. These beads had bigger weight and lower calcium content and force at crush. Interestingly, S beads showed characteristics from both groups. S beads had spindle shape and smaller weight like B<sub>C</sub> and E beads, whereas their calcium content was more similar to the group of ES and C<sub>ES</sub> beads, although their force at crush was in-between both groups.

Table 3.2 Physical characteristics of calcium alginate beads

Beads	Calcium Content [mM]	Aspect ratio	Weight [mg]	Crushing Force [N]
B <sub>C</sub>	1.51	2.06±0.76	0.93±0.25	1.48±1.22
E	1.55	1.67±0.52	0.93±0.09	1.73±1.49
ES	0.47	1.4±0.40	1.82±0.35	1.02±1.62
S	0.65	1.67±0.56	1.01±0.19	1.19±0.58
C <sub>ES</sub>	0.58	1.28±0.23	2.00±0.41	1.06±0.60

B<sub>C</sub> (blank control beads), E (beads containing entrapped lactobacilli);  
 ES (beads containing both entrapped and surface lactobacilli);  
 S (beads containing surface lactobacilli);  
 C<sub>ES</sub> (beads containing concentrated entrapped and surface lactobacilli).

The morphology of the two groups was also distinct (Figure 3.2, a-e). The internal and external surfaces of B<sub>C</sub> and E beads were rough, while the ones of ES, C<sub>ES</sub> and S beads were smooth. Large colonization on the entire surface was observed particularly in C<sub>ES</sub> beads, followed by ES beads. Rare lactobacilli gathered in groups were found only on the external surfaces of S beads.

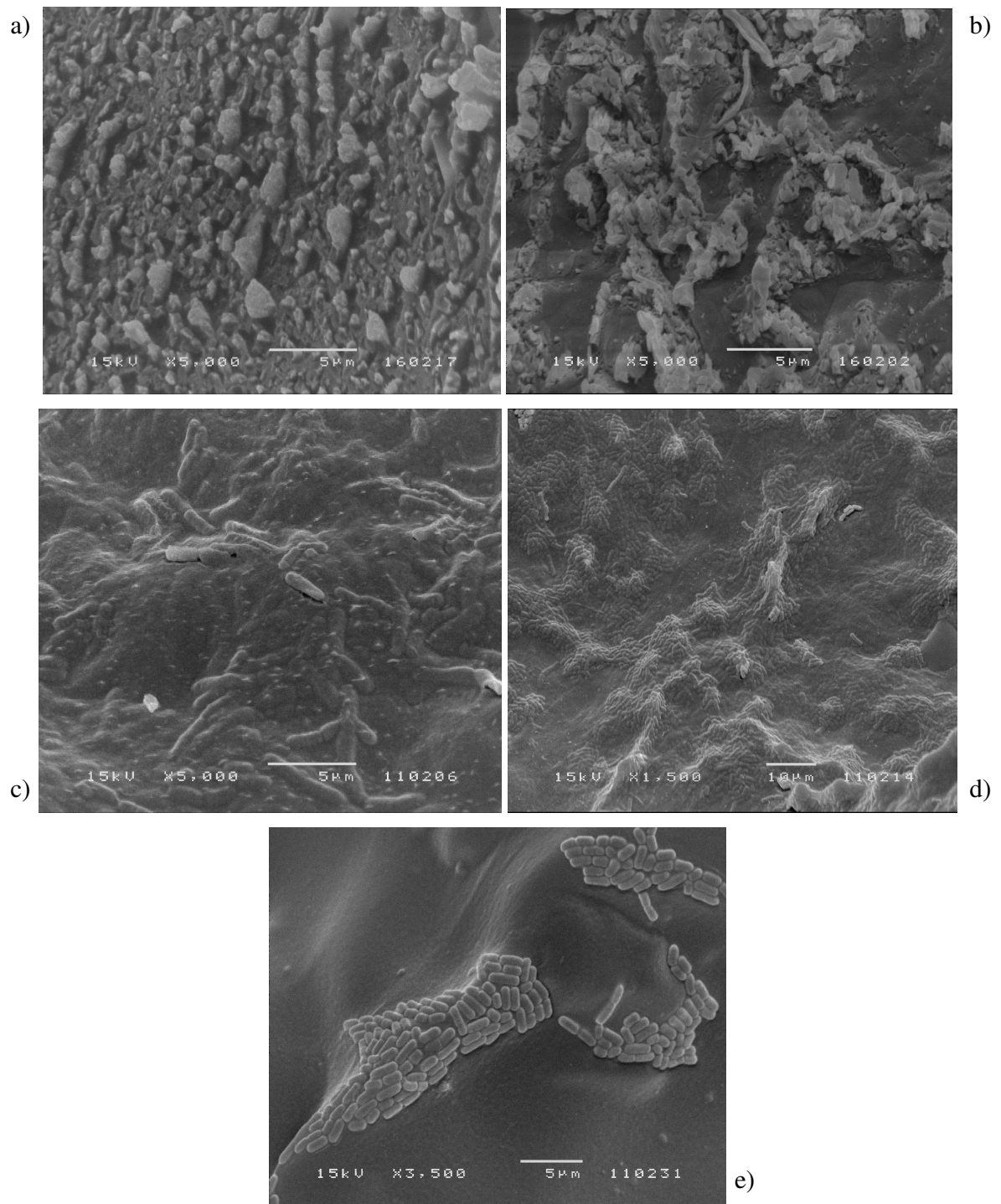


Figure 3.2 Scanning electron photomicrographs of the external surface of a) blank bead ( $B_C$ ); b) bead containing entrapped lactobacilli (E); c) bead containing entrapped and surface lactobacilli (ES); d) bead containing concentrated entrapped and surface lactobacilli ( $C_{ES}$ ); e) bead containing surface lactobacilli (S).

### 3.3.2.2 Release characteristics

Release of the total quantity of lactobacilli was observed in the first 10 min from S beads (Figure 3.3). The released lactobacilli in the first 10 min from the other types of beads corresponded to 1.4% or  $10^9$  cfu/g for  $C_{ES}$  beads; 2.1% or  $10^7$  cfu/g for E beads; and 2.5% or  $10^8$  cfu/g for ES beads (Figure 3.3). ES, E and  $C_{ES}$  beads released their total quantity of lactobacilli as follows: up to the second, fourth and tenth hour, respectively.

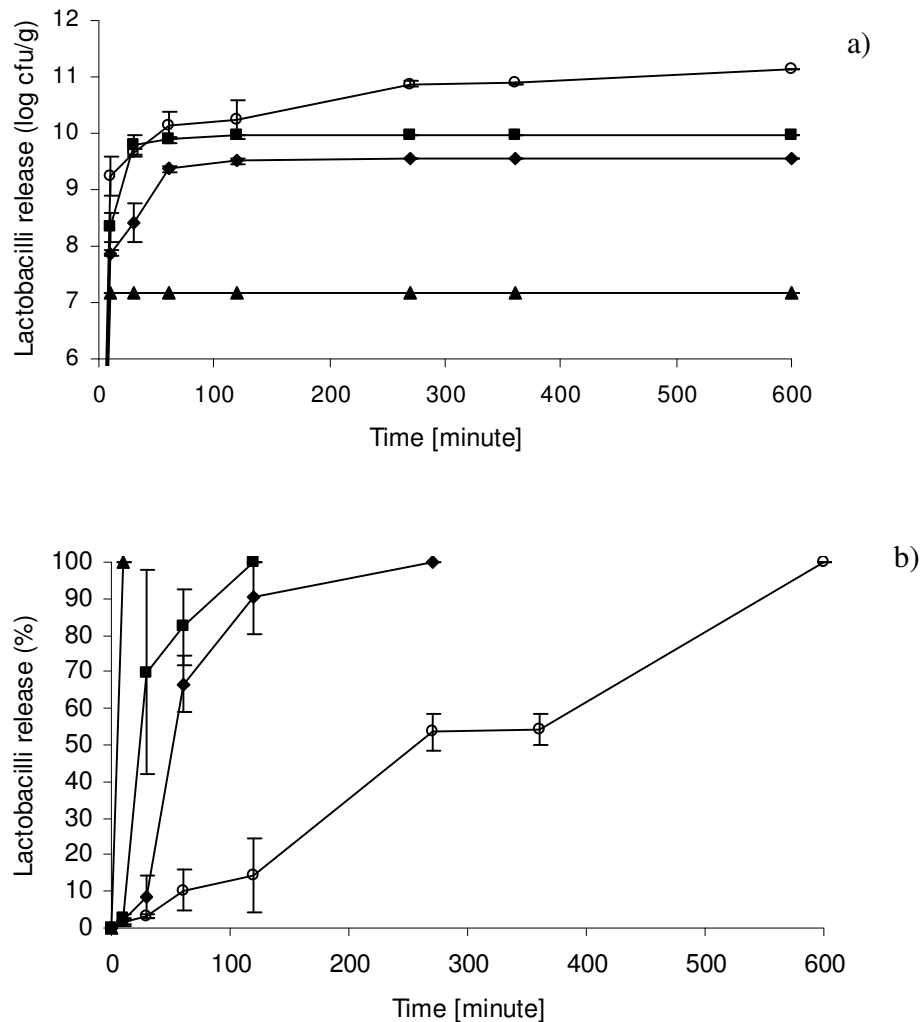


Figure 3.3 Release of lactobacilli from beads, represented as log cfu/g (a) and in % (b), (beads containing  $\blacklozenge$  entrapped (E),  $\blacksquare$  entrapped and surface (ES),  $\blacktriangle$  surface (S);  $\circ$  concentrated entrapped and surface lactobacilli ( $C_{ES}$ )).

### 3.3.2.3 Viability and antibacterial activity

Tests of the antibacterial activity of beads containing entrapped lactobacilli (E beads) were performed on agar plates by well diffusion method. Immediately after immobilization *L. plantarum*, LGG and *L. bulgaricus* loaded beads demonstrated decreased (i.e. smaller inhibition zones) or no antibacterial activity. However, immobilisation did not influence the antibacterial activity of *L. lactis* (data not shown). Freeze-drying of E beads led to low survival rates of *L. bulgaricus* (from  $2.3 \times 10^8$  to  $1.5 \times 10^6$  cfu/ml) and *L. lactis* ( $1.0 \times 10^9$  to  $<10^2$  cfu/ml) (Figure 3.4a) and lack of inhibitory activity.

Incubation of E beads in MRS broth led to proliferation of lactobacilli to the surface of the beads and surface colonization (ES beads) (Figure 3.2c). ES beads showed a better survivability of lactobacilli, particularly of *L. bulgaricus* and *L. lactis*, during freeze-drying compared to E beads (Figure 3.4). The viability decrease of lactobacilli in ES beads due to freeze-drying was  $\leq 1$  log unit (from  $3.4 \times 10^9$  to  $3.5 \times 10^8$  cfu/ml for *L. plantarum*, from  $1.1 \times 10^9$  to  $4.9 \times 10^8$  cfu/ml for LGG, from  $1.7 \times 10^8$  to  $1.8 \times 10^7$  cfu/ml for *L. bulgaricus* and from  $1.0 \times 10^9$  to  $5.2 \times 10^8$  cfu/ml for *L. lactis*) (Figure 3.4b). Preserved antibacterial activity of ES beads was first observed on agar and then confirmed in broth. Figure 3.5 represents the antibacterial activity of ES beads as a function of lactobacilli viability and compares it to the antibacterial activity of lactobacilli cultures before processing. The antibacterial activity of *L. lactis* against *S. aureus*, *P. aeruginosa* and *K. pneumoniae* in MH broth remained unchanged after processing and inhibition was obtained by  $10^7$  *L. lactis* cells. Processing influenced little the antibacterial activity of *L. plantarum* and LGG against *E. coli* and *K. pneumoniae* in MH broth and inhibition was obtained by  $10^8$  lactobacilli cells. A change in activity after processing of *L. lactis* against *E. coli* and of *L. plantarum* and LGG against *S. aureus*, *E. faecalis* and *P. aeruginosa* was reflected by 1 log unit increase (from  $10^7$  to  $10^8$  cfu/ml) of the lactobacilli cells necessary to inhibit the multi-resistant strains. More pronounced change in activity was marked only when *L. lactis* beads were used against *E. faecalis*. Inhibition was observed by  $10^9$  immobilized *L. lactis* cells versus  $10^7$  non-processed *L. lactis* cells.

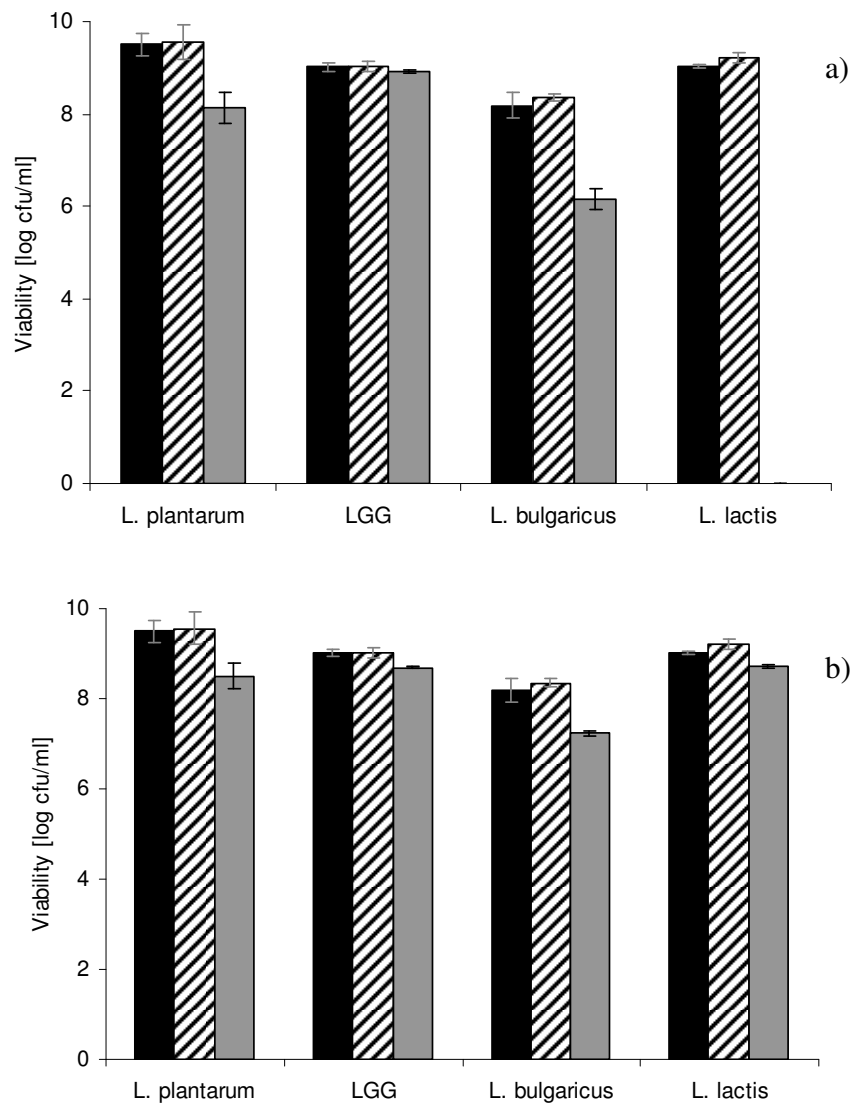


Figure 3.4 Alginate beads, containing a) entrapped (E) and b) entrapped and surface lactobacilli (ES). Number of viable lactobacilli (mean and SD, n=3) in overnight cultures (black bar), wet alginate beads (dashed bar) and dry alginate beads (grey bar).

By analogy with the overnight cultures, the antibacterial activity of the immobilized lactobacilli against *S. aureus* and *K. pneumoniae* was promoted by MRS broth, reflected by 1 to 2 log units reduction of the lactobacilli cells necessary to obtain inhibition (from  $10^7$ - $10^8$  in MH broth to  $10^6$ - $10^7$  cfu/ml in MH+MRS broth). The influence of MRS was again most significant against *P. aeruginosa* decreasing 3 to 4 log units the number of lactobacilli cells necessary to inhibit the organism (from  $10^8$  to  $10^4$  cfu/ml for *L.*

*plantarum*; from  $10^8$  to  $10^5$ cfu/ml for LGG; from  $10^7$  to  $10^4$ cfu/ml for *L. lactis*) (Figure 3.5). Independent of the broth medium the immobilized lactobacilli were only active against *E. coli* and *E. faecalis* incubated at high cell densities, i.e.  $10^8$  and  $10^9$ cfu/ml, respectively. The antibacterial activity of the immobilized LGG and *L. lactis* against *E. faecalis* could not be determined late in storage (Figure 3.5), which could be related to a 71% viability decrease, i.e. from  $9.0 \times 10^9$  cfu/g after freeze-drying to  $2.5 \times 10^9$  cfu/g (Figure 3.6a). Similarly, the antibacterial activity of *L. bulgaricus* against all multi-resistant strains, except *P. aeruginosa* in the combined medium of MH with MRS, could not be determined. Lactobacilli viability in ES beads decreased  $\leq 1$  log unit until the end of storage (from  $9.0 \times 10^9$  to  $3.7 \times 10^9$ cfu/g for *L. plantarum*;  $8.5 \times 10^9$  to  $2.5 \times 10^9$ cfu/g for LGG;  $9.0 \times 10^9$  to  $2.5 \times 10^9$ cfu/g for *L. lactis*;  $1.8 \times 10^8$  to  $1.9 \times 10^7$ cfu/g *L. bulgaricus*) (Figure 3.6a).

The alginate beads containing surface lactobacilli only (S beads), reached cell concentrations of  $10^8$ cfu/ml at the end of incubation. However, their viability dropped 2 log units ( $10^6$ cfu/ml) during freeze-drying, which was not sufficient to be inhibitory active.

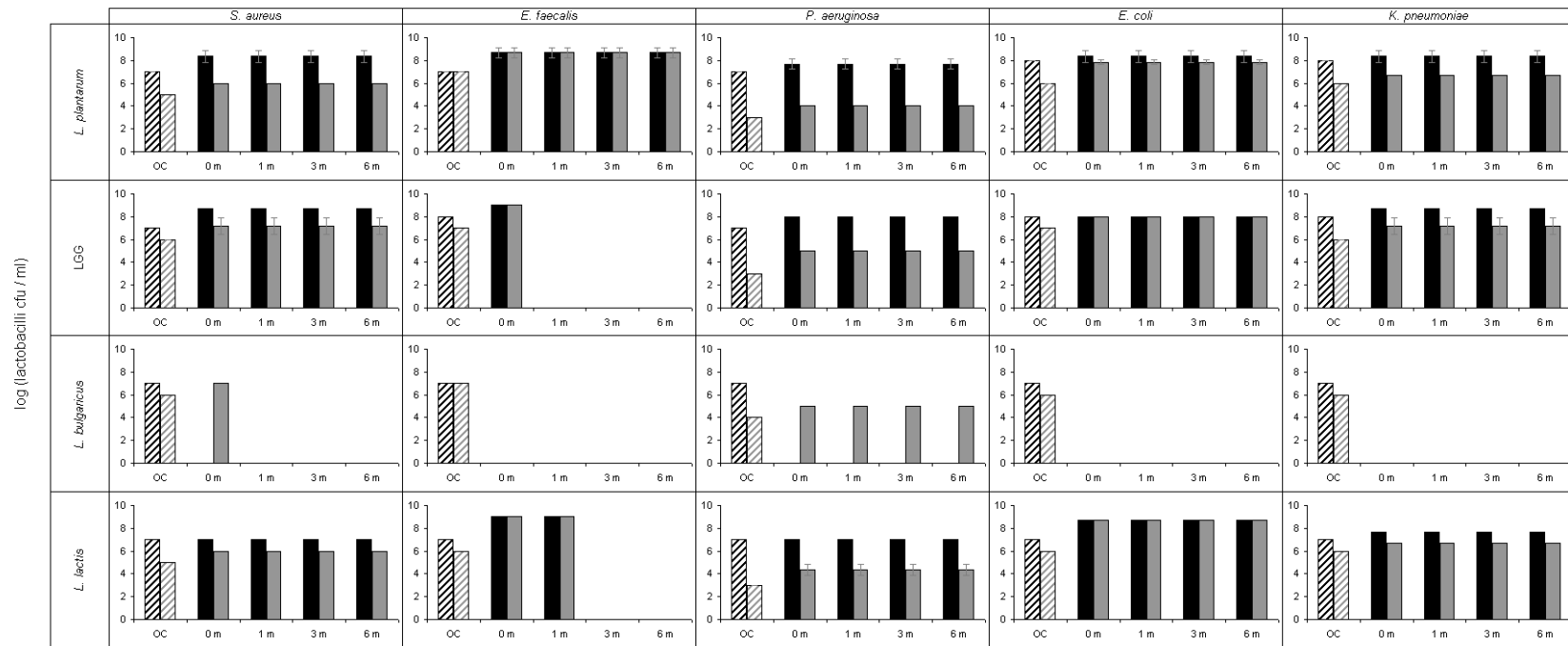


Figure 3.5 Antibacterial activity of lactobacilli (mean and SD, n=3) in beads containing entrapped and surface lactobacilli (ES beads), represented as the logarithm of the minimal number of lactobacilli colony forming units (cfu) necessary to inhibit completely the growth of the multi-resistant strains determined during six months of storage at 4°C, with the overnight cultures (OC) as controls. (▨): MH; (■): MH+MRS.



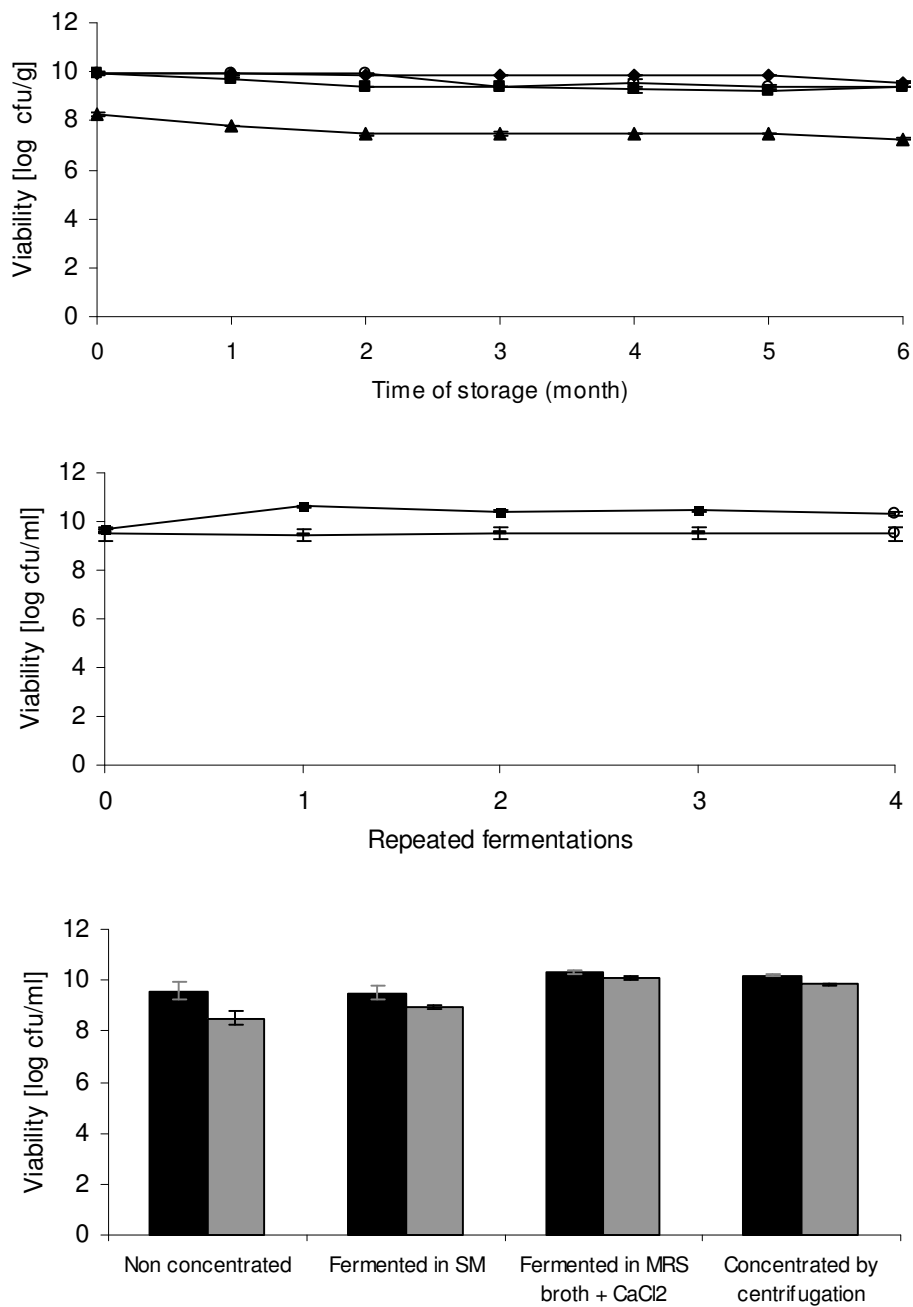


Figure 3.6 Viability of lactobacilli (mean and SD, n=3) in different conditions  
a) lactobacilli in dry alginate ES beads throughout 6 months of storage at 4°C for *L. plantarum* (●), LGG (■), *L. bulgaricus* (▲) and *L. lactis* (○);  
b) *L. plantarum* in alginate beads during four repeated fermentations: three in skim milk (-) and in MRS broth with 1% CaCl<sub>2</sub> (■) and a final fermentation in MRS broth (○);  
c) *L. plantarum* in non-concentrated and concentrated beads before (●) and after freeze-drying (■).

The observed relationship between antibacterial activity and viability suggested that concentrating the immobilized cells could be an approach for optimization. CaCl<sub>2</sub> was added to MRS to improve the beads stability (Morin et al., 1992), evading softening or dissolution of the beads during repeated fermentations. Fermentations performed in MRS broth supplemented with 1% CaCl<sub>2</sub> led to an increase of *L. plantarum* cell concentrations ( $5 \times 10^9$  to  $2 \times 10^{10}$ cfu/ml), while in skim milk the number of cells remained unchanged (Figure 3.6b). Concentrating *L. plantarum* immobilized suspensions made the microorganism more resistant to freeze-drying (from  $2 \times 10^{10}$ cfu/ml before freeze-drying to  $1.2 \times 10^{10}$ cfu/ml or  $2.2 \times 10^{11}$ cfu/g after freeze-drying for beads concentrated by fermentation in MRS with CaCl<sub>2</sub>; from  $1.5 \times 10^{10}$ cfu/ml to  $7 \times 10^9$ cfu/ml ( $1.4 \times 10^{10}$ cfu/g) for beads concentrated by centrifugation) (Figure 3.6c). When the antibacterial activity of the concentrated beads was tested in MH+MRS, the concentration of *L. plantarum* in beads was reflected by a decrease (about 1/10) of the number of beads required to reach inhibition. However, in MH medium the relationship between the antibacterial activity and the viability of the concentrated beads was different from non-concentrated beads (Figure 3.7), i.e. the concentrated beads needed more cells to inhibit the multi-resistant clinical isolates in MH broth. Consequently, the process of concentration was not reflected by a decrease of the number of beads required to reach inhibition.

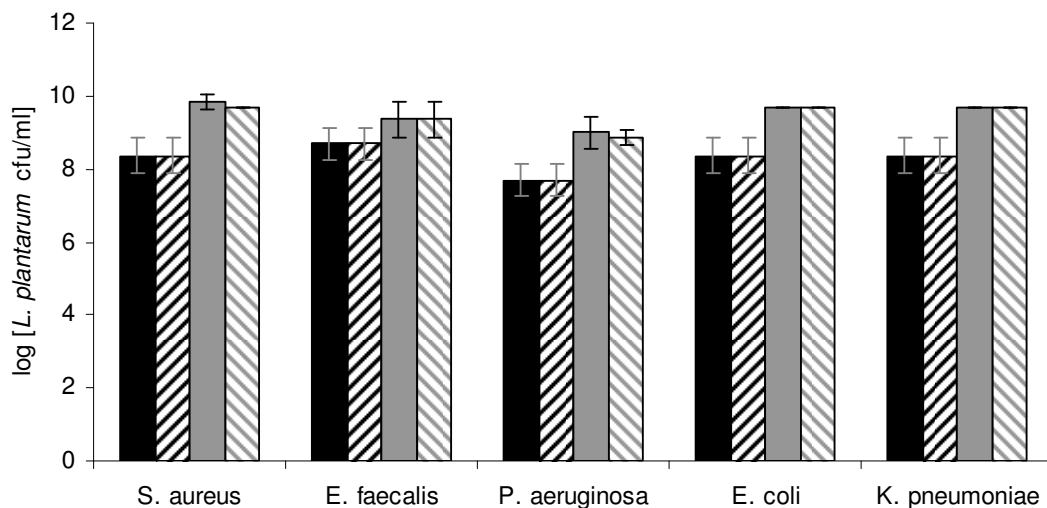


Figure 3.7 Antibacterial activity (mean and SD, n=3) of *L. plantarum* non-concentrated ES beads (black bar), fermented in skim milk (dashed black bar), fermented in MRS broth with 1% CaCl<sub>2</sub> (grey bar) and concentrated by centrifugation beads (dashed grey bar), represented as logarithm of the minimal number of colony forming units (cfu) of *L. plantarum* necessary to inhibit completely the growth of the multi-resistant strains, determined in MH broth.

### **3.4 Discussion**

#### **3.4.1 Antibacterial activity of lactobacilli before processing**

According to the results of the well diffusion method the whole lactobacilli cultures had the widest antibacterial spectrum compared to culture filtrates and washed cells. It was due to the fact that lactobacilli antibacterial activity against the Gram-positive multi-resistant strains was cell related, while against the Gram-negative strains referred to antibacterial substances excreted in the culture filtrates.

The observation that lactobacilli culture filtrates were active against *S. aureus* only on MH agar, suggested that the activity of lactobacilli whole cultures and washed cells on MRS agar against the organism could not be due to the antibacterial substances excreted into the broth, but could be mediated by production of antibacterial substances during the co-growth of the antagonistic strains on MRS agar. Similar interpretation could be made concerning lactobacilli activity against *E. faecalis*. The fact that lactobacilli cells were necessary for the inhibition of *E. faecalis* and inhibition occurred on MRS agar only suggested that the antibacterial activity against *E. faecalis* could be related to antibacterial substances produced during the growth of lactobacilli on MRS agar and not in MRS broth. Further proof of this suggestion was obtained by the similar sizes of the inhibition zones produced by either whole cultures or washed cells. Strains exhibiting antagonistic effects only on agar but not in liquid medium have been described in literature (Schillinger and Lücke, 1989).

The antibacterial activity against the Gram-negative multi-resistant isolates could be mediated by antibacterial substances excreted during the growth of lactobacilli in MRS broth. The results were in accordance to the conclusions reached by Valdez et al., 2005, who observed inhibition of *P. aeruginosa* PA100 by whole cultures and culture filtrates of *L. plantarum* ATCC10241. Moreover, these researchers showed that *L. plantarum* ATCC10241 can inhibit the production of *P. aeruginosa* quorum-sensing signal molecules and the virulence factors elastase and biofilm formation controlled by these signal molecules.

The fact that lactobacilli washed cells were active on MRS agar and not on MH suggested that, although MH permitted the growth of lactobacilli it did not promote their

antibacterial activity. This observation was confirmed by the sizes of the inhibition zones. The sizes of the inhibition zones on MH agar produced by whole cultures (mean value of 12mm) were corresponding to the sizes of the inhibition zones produced by the culture filtrates (mean value of 12mm), while the inhibition zones of the whole cultures on MRS agar were larger (mean value of 20mm).

A relationship between lactobacilli antibacterial activity and viability has been found by the microdilution test. A density of  $10^7$ - $10^8$  cells/ml was necessary to observe inhibition in MH broth. Promotion of the lactobacilli antibacterial activity against the multi-resistant strains, most significant against *P. aeruginosa*, was observed in the presence of MRS in the microdilution medium (Figure 3.1). This phenomenon could be due to enhanced production of highly active bacteriocins by MRS medium. In a different study (Todorov and Dicks, 2005), despite of the relatively good cell growth, low bacteriocin activity ranging from 200 to 800 AU/ml (AU=arbitrary units) was recorded as a result of growing *L. plantarum* ST23LD in M17 broth, BHI broth and soy milk. *L. plantarum* ST23LD produced the highest activity of its bacteriocin (25600 AU/ml) when grown in MRS broth.

### **3.4.2 Lactobacilli loaded calcium alginate beads**

The observed antibacterial activity of the lactobacilli strains against multi-resistant strains allowed us to consider the development of a formulation containing overnight lactobacilli cultures with preserved and stable viability and antibacterial activity. The composition of the formulation had to be poor, containing no nutrients which could boost the growth of the multi-resistant strains and on the other hand it had to protect the lactobacilli cells during processing and preserve their antibacterial activity. Following the previous discussion, the presence of MRS could be favourable in a formulation containing lactobacilli.

When the cell-alginate suspension was added to  $\text{CaCl}_2$  solutions, calcium alginate beads were formed. However, alginate beads are not inert but are constantly in interaction with the medium in which they are put (Velings and Mestdach, 1995). Therefore, the morphology of the beads before and after incubation in MRS broth was quite different

(Figure 3.2: B<sub>C</sub> and E beads vs. ES, C<sub>ES</sub> and S beads). It might be due to the interaction between the lactobacilli cells and the calcium alginate matrix. The rough internal and external surfaces of beads (B<sub>C</sub> and E beads) were transformed into smooth surfaces during the proliferation of lactobacilli in calcium alginate gel (ES, C<sub>ES</sub> and S beads). The proliferation of lactobacilli resulted in a decrease of the calcium content in the beads (Table 3.2: B<sub>C</sub> and E beads vs. ES, C<sub>ES</sub> and S beads), which could be due to production of lactic acid by the bacteria during incubation of beads in MRS broth, which competes with the alginate for calcium (Morin et al., 1992). The decrease in calcium in the calcium–alginate network in ES, S and C<sub>ES</sub> beads resulted in a destabilising effect reflected by lower forces at crush (Table 3.2). Lower calcium content is related also to network formation with increased space occupied by alginate and therefore increased bead volume and on the other hand to higher medium absorption into the beads (Velings and Mestdach, 1995). Higher MRS absorption into ES and C<sub>ES</sub> beads might have contributed to obtain higher values of dry beads weight. However, this factor is probably of minor significance, as proven by S beads. Although S beads had lower calcium content, comparable to ES and C<sub>ES</sub> beads, their weight was smaller, similar to B<sub>C</sub> and E beads. Other factors that might have resulted in increase of ES and particularly C<sub>ES</sub> beads weight are the biomass increase (Trauth et al., 2001) and the accumulation of metabolic products in beads (Lacroix et al., 2005). Furthermore, S, E and B<sub>C</sub> beads shrank during freeze-drying and their shape changed from spherical to prolate ellipsoid, while the shape of ES and C<sub>ES</sub> beads remained more spherical. This fact suggests that the creation of networks between lactobacilli during their proliferation to beads surface could have helped avoiding bead shrinkage during freeze-drying and preserving bead shape.

Several factors influenced lactobacilli release from beads. The presence of surface lactobacilli in alginate beads explained the observed burst effect: immediate release after contact with PBS of the total lactobacilli content from S beads, and of 1.4 to 2.5% from the other types of beads (Figure 3.3). The decreased crosslinking density with calcium and the consequent increase of porosity (Wan et al., 2008) and lower mechanical strength, reflected by lower forces at crush, could explain the quicker lactobacilli release from ES beads than from E beads (Figure 3.3).

The presence of both surface and entrapped lactobacilli was found to promote higher survival rates during freeze-drying. The survival rates were higher in ES and C<sub>ES</sub> than in E and S beads (Figure 3.4a,b and 3.6c). In general, surviving fraction of the microorganisms during freeze-drying increases with the increasing biomass concentration, and this is attributed to the mutual shielding effect of the microorganisms against the severe conditions of the external medium (Bozoglu et al., 1987). In the present study, 10<sup>9</sup>-10<sup>10</sup>cfu/ml was found to be optimal initial bacterial cell concentration resulting in the highest survivability during freeze-drying (ES and C<sub>ES</sub> beads). On the other hand, 10<sup>8</sup>cfu/ml was found to be too low (S beads): the cell viability might be lowered through lack of the protective effects generated by mutual contact of the cells due to lack effective networks (Souzu, 2004).

Immobilization of lactobacilli in calcium alginate to obtain beads containing entrapped lactobacilli (E beads) and subsequent freeze-drying led to partial or complete loss of lactobacilli antibacterial activity. As discussed, high lactobacilli viability was a prerequisite to observe antibacterial activity. On the other hand, possible reasons to explain the lack of activity when high viability was preserved (*L. plantarum* and LGG beads) could be due to a slow release of antibacterial substances or cells from the beads (George and Abraham, 2006), as well as, a change in activity of lactobacilli and their metabolic products due to process of desiccation (Viernstein et al., 2005). Incubation of beads in MRS broth to obtain ES beads contributed to preserve lactobacilli antibacterial activity. This could be due to 1) the better survivability of lactobacilli during freeze-drying; 2) the presence of surface lactobacilli leading to burst effect of cells; and to 3) the decrease of calcium from the calcium-alginate network of the beads, which could have contributed to quicker release of not only cells (Figure 3.3) but also of antibacterial substances, important for the promotion of the antibacterial effects of the lactobacilli. On the other hand, beads containing surface lactobacilli (S beads) showed lower survival rates during freeze-drying and consequently no inhibition. Concentrated alginate beads (C<sub>ES</sub>) did not have any advantage referring lactobacilli antibacterial activity compared to ES beads. A possible explanation could be that the increase of the number of lactobacilli cells in the concentrated beads could have not corresponded to an equivalent increase of

the antibacterial substances. Wan et al., 1995, found that in repeated batch fermentations, immobilized cells produced less bacteriocin than free cells.

### **3.5 Conclusions**

The study has confirmed the possibility of immobilization of different lactobacilli strains in calcium alginate by transformation of cell cultures into solid beads. Nevertheless a decrease of antibacterial activity and viability due to immobilization and freeze-drying was observed, this decrease could be reversed with further incubation of the dosage forms in MRS medium. The consequent proliferation of the entrapped lactobacilli to the beads surface resulted in a significant change in the physicochemical properties of beads showing smoother surface morphology, more spherical shape, bigger weight, lower calcium content and force at crush.

The study showed that lactobacilli cultures at high cell concentrations of  $10^8$ cfu/ml had wide antibacterial spectrum against both Gram-positive and Gram-negative multi-resistant clinical isolates. Furthermore, the preservation of lactobacilli antibacterial activity during processing and storage was related to the preservation of their viability.

## **CHAPTER 4**

### **ALGINATE FILMS CONTAINING *LACTOBACILLUS* *PLANTARUM* AS WOUND DRESSING FOR PREVENTION OF BURN INFECTION: PREPARATION, IN VITRO AND IN VIVO EVALUATION**



## **4.1 Introduction**

The experience in topical bacteriotherapy in burn patients is limited (see 1.1.2.8). However promising results (Peral et al., 2009) suggest that lactobacilli could be a novel and useful approach for local prevention and treatment of burn wound infections.

## **4.2 Materials and Methods**

### **4.2.1 Materials**

The used materials (glycerol, sodium chloride, calcium chloride, ethylenediaminetetraacetic acid, sodium alginate, de Man-Rogosa-Sharpe agar and broth (MRS), Mueller-Hinton (MH) and Cetrimide agar) were listed in 3.2.1.

### **4.2.2 Methods**

#### **4.2.2.1 Bacterial strains and culture conditions**

*Lactobacillus plantarum* ATCC 8014 was grown overnight at 37°C on MRS agar and in broth. A VIM-2-metallo- $\beta$ -lactamase-producing *Pseudomonas aeruginosa* FFUL 1396, isolated from abdominal wound of a patient at a Central Hospital of Lisbon, was grown overnight at 37°C on its selective agar medium Cetrimide.

*P. aeruginosa* was chosen as an indicator strain in this study based on its prevalency in burn patients, resulting in severe complications and mortality reaching up to 40-50% (Estahbanati et al., 2002). Treatment of *P. aeruginosa* infections is difficult due to the high frequency of *P. aeruginosa* resistance (Pournaras et al., 2009; Estahbanati et al., 2002).

#### **4.2.2.2 Preparation and in vitro evaluation of *L. plantarum* loaded calcium alginate films**

##### **4.2.2.2.1 Preparation of *L. plantarum* loaded calcium alginate films**

Sodium alginate (1g) was dissolved in *L. plantarum* overnight cultures (50ml). 9.5ml of the cell-alginate suspension was poured into a Petri plate (5.5cm diameter) containing 3ml of twofold increasing CaCl<sub>2</sub> concentrations (0.05-12.8M). The films were cured for 15h at 4°C in the calcium solutions to reach steady state equilibrium (Velings and Mestdagh, 1995). Ellipse shaped films were obtained. The time of film formation was recorded and film dimensions were measured with Calliper. After curing each film was incubated in MRS broth at 37°C/4h to obtain proliferation of the entrapped lactobacilli to the films surfaces. Blank films, containing only MRS broth and no lactobacilli, were used as controls.

All types of films were frozen at -80°C for 24h and dried under vacuum at room temperature, 0.035mbar for 24h (Christ Alpha 1-4, B. Braun Biotech International, Germany). The films produced were kept over silica gel, at 4°C, for period of 6 months. These films were considered for further characterisation.

##### **4.2.2.2.2 Scanning electron microscopy**

The morphology of samples from the lactobacilli loaded and blank films was examined as described in 3.2.2.4.2.

##### **4.2.2.2.3 Calcium content**

The calcium concentration in the films is paramount to their formation and physico-chemical properties; therefore the amount of calcium remaining in the film provides information of the properties of the films. It was determined according to 3.2.2.4.3.

##### **4.2.2.2.4 Water absorption capacity**

Film strips (dimensions 3.2 x 2.2 x 0.4 cm) were suspended in 50ml of phosphate-buffered saline (PBS) (pH 7.4) at room temperature. At specified time intervals (ranging

between 1 minute and 48 hours, depending on the film), the films were taken out and the excess of water removed carefully with filter paper, then weighed immediately. The swelling ratio of the films was calculated as the ratio between the weight of a swollen sample to that of a dry sample.

#### 4.2.2.2.5 Mechanical properties

The mechanical properties of film strips (3.2 x 2.2 x 0.4 cm) were measured using a texture analyzer (TA.XTPlus, Stable Micro Systems, UK) equipped with a 5 kgf (approx. 50N) load cell and fit with a three-point bending rig apparatus. Film strips were hydrated for 5 minutes, to allow comparison between different films, and placed over the supports, positioned at a distance of 7 mm. The upper blade moved down equidistant from the two lower supports at a speed of 3 mm/s. The breaking force was measured and the tensile strength (Davies and Newton, 1995) and Young's modulus (Rowe and Roberts, 1995) were calculated as follows:

$$\sigma_f = \frac{3Fl}{2bd^2} \quad \text{eq. 4.1}$$

$$E = \frac{Fl^3}{4\xi d^3 b} \quad \text{eq. 4.2}$$

where,  $\sigma_f$  is the tensile fracture stress,  $F$  is the load at fracture,  $l$  is the distance between the supports,  $b$  is the film width,  $d$  is the film depth,  $E$  is Young's modulus,  $\xi$  is the central deflection.

#### 4.2.2.2.6 Bioadhesion properties

The bioadhesive strength of the films was evaluated according to Sezer *et al.* [17] (2007). The measurement was conducted with a texture analyzer (TA.XT Plus, Stable Micro Systems, UK) fit with a 5 kgf load cell and bioadhesion test apparatus which included a cylindrical Perspex probe. Chicken back skin was used as a model tissue, after the removal of all fats and debris. The dermal tissue was fitted on the bioadhesion test

apparatus, and then 100µl of demineralised water was applied on the surface of the tissue prior to measurements at 37°C. The film was cut into small cylinders shape and attached to probe with double sided adhesive. The probe was lowered onto the surface of the tissue with a constant speed of 1mm/s and constant force of 1N applied. After keeping in contact for 30 seconds, the probe was then moved vertically upwards at a constant speed of 1mm/s. Work of adhesion ( $J/m^2$ ) and peak detachment force ( $N/m^2$ ) were calculated from the force *versus* displacement graphic.

#### **4.2.2.2.7 Release of lactobacilli**

The release of lactobacilli from films was studied as described in 3.2.2.4.5.

#### **4.2.2.2.8 Viability of lactobacilli**

The lactobacilli viability was determined before and after immobilisation and freeze-drying and throughout 6 months of storage by the agar plate method. 20 mg samples were collected from the centre and the periphery of films. These results were averaged for each sample and then the mean and standard deviation calculated: low standard deviation reflected little variation between the centre and the periphery of the film. Calcium alginate was dissolved in sterile sodium citrate solution (1%, pH 6.0) followed by serial dilutions made in sterile water and plated in duplicate on MRS agar.

#### **4.2.2.2.9 Antibacterial activity**

Micro- and macrodilution broth methods (see 3.2.2.3 and 3.2.2.4.7) were used to test the in vitro antibacterial activity of *L. plantarum* before and after processing into calcium alginate films and during 6 months of storage. Samples were analyzed at 6<sup>th</sup>, 8<sup>th</sup>, 10<sup>th</sup> and 24<sup>th</sup>h to determine the time necessary for the inhibition of *P. aeruginosa*.

#### 4.2.2.3 In vivo experiments

Studies were carried out using 25 male Wistar rats (from 292 to 384g, Harlan Ibérica, Barcelona, Spain). Rats received a standardized diet and water *ad libitum* in a 12 h light/darkness cycle at 22°C. After the stabilization period, rats were randomly allocated into five groups: group NBc: non-burned and non-treated control rats ( $n=2$ ); group Bc: non-treated burned control rats ( $n=6$ ); group BL: the burned skin was covered with films containing *L. plantarum*, obtained with 1.6 M CaCl<sub>2</sub>, ( $n=3$ ); group BPs: the burned skin was contaminated with *P. aeruginosa* ( $1 \times 10^8$ cfu/ml) by sterile cotton swabs ( $n=7$ ); group BPsL: the burned skin was contaminated with *P. aeruginosa* and covered with films containing *L. plantarum* ( $n=7$ ). To function effectively alginate dressings need to absorb exudate from the wound, but the burn wounds of rats are dry. Therefore films were hydrated with sterile phosphate buffered saline (1ml / 100mg of film) before application.

All animals were subjected to the surgical procedures as follows. All rats were anesthetized with sodium pentobarbital (Eutasil™, 60 mg/kg, intra peritoneal; Sanofi Veterinária, Algés, Portugal). Depth of anesthesia was assessed by pedal reflex and maintenance doses were given as required. Anesthetized rats were shaved (dorsum) and placed onto a thermostatically controlled heating mat (Harvard Apparatus Ltd, U.K.) and body temperature maintained at  $37 \pm 1^\circ\text{C}$  by means of a rectal probe attached to a homeothermic blanket. A tracheotomy was performed to maintain airway patency and to facilitate spontaneous respiration. The thermal injury procedures were modified from those previously described (Walker and Mason, 1968): a 30% third degree skin burn was induced by immersing dorsal shaved skin in 96-99°C water for 10 seconds using a synthetic foam template after surgical procedure. Group NBc was submitted to identical procedures as the other groups except that room temperature water was used instead of heated water. After burn, animals were dried and placed over the heating mat. Animals were sacrificed 8 hours after burn injury by overdose of the anaesthetic. The experimental time (8 hours) was the time necessary for inhibition of *P. aeruginosa* in vitro (see 4.3.1).

At the end of the experimental period, blood was collected through cardiac puncture and placed into a serum SST® gel and clot activator (BD Vacutainer<sup>(R)</sup> SST™, Becton Dickinson, Meylan, France). After this, lung, liver, spleen, kidney, intestine and burned

skin (approximately 1 cm<sup>2</sup>) samples were collected. To determine the colony forming unit (cfu)/g of tissue, the specimen was homogenized in MH broth (1:10) and then cultured onto non-selective MH agar and selective media: Cetrimide agar for *P. aeruginosa* and MRS agar for *L. plantarum*. The inoculated plates were incubated at 37°C and read after 24 to 48h. The blood samples and the homogenized tissues were further incubated at 37°C for 24h. 100µl from the overnight cultures were plated on Cetrimide and on MRS agar to confirm if *P. aeruginosa* or *L. plantarum* cells less than the level of detection of the direct assay have passed into the blood and the organs of the animals (group BL, BPs and BPsL).

Simultaneously serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and urea were quantified and alterations observed were compared to the control groups NBc and Bc. The results were expressed as the mean ± SE and were compared using a one-way factorial ANOVA test, followed by a Bonferoni post-hoc-test using a Graph Pad Prism Statistical Package (version 4.0, USA). A *p* value less than 0.05 was considered to be statistically significant on evaluating the differences between the groups of animals (Mitsui et al., 1999).

### 4.3 Results

#### 4.3.1 Preparation and in vitro evaluation of *L. plantarum* loaded calcium alginate films

The production of a film was observed with a minimal calcium concentration of 0.8 M CaCl<sub>2</sub>. Below this value films did not show an adequate structure falling apart. On the other hand, by increasing the concentration of calcium in the calcium chloride solution, the time for films formation decreased together with a smaller volume observed (Table 4.1).

Table 4.1 Visual inspection of the forming films (mean and SD).

CaCl <sub>2</sub> formation solution [M]	0.8	1.6	3.2	6.4	12.8
Time for film formation [h]	3	2.5	1	1	0.25
Film major and minor axis [mm]	42.5±0.7 / 47.4±1.2	43.4±1.3 / 46.0±1.9	43.3±1.2 / 46.0±1.3	43.0±1.3 / 45.0±1.5	43.0±1.2 / 45.0±1.3
Film thickness [mm]	5.23±0.26	5.08±0.19	5.08±0.19	4.65±0.19	4.35±0.15

The aqueous solution of CaCl<sub>2</sub> used in a concentration up to 3.2 M did not affect *L. plantarum* viability. However, higher concentrations (6.4 M) led to both a lack of uniformity, i.e., *L. plantarum* cell concentration in the periphery of the film ( $2 \times 10^7$  cfu/ml) was lower compared to the cell concentration in the central part of the films ( $8 \times 10^8$  cfu/ml) (higher standard deviation bars) (Figure 4.1a, immobilization) and to a viability decrease lower than the limit of detection noted with 12.8M CaCl<sub>2</sub> (Figure 4.1a, immobilization). Freeze-drying resulted in a decrease of approximately 1 log unit: from  $4.7 \times 10^9$  to  $2.4 \times 10^8$  cfu/ml,  $3.3 \times 10^9$  to  $3.0 \times 10^8$  cfu/ml,  $1.8 \times 10^9$  to  $3.0 \times 10^8$  cfu/ml of *L. plantarum* viability in films obtained with 0.8, 1.6 and 3.2M CaCl<sub>2</sub>, respectively. Stable viability of bacteria during six months of storage was observed in the same films ( $4.7 \times 10^9$  to  $5.5 \times 10^9$  cfu/g;  $5.9 \times 10^9$  to  $1.4 \times 10^9$  cfu/g;  $5.9 \times 10^9$  to  $2.7 \times 10^9$  cfu/g) (Figure 4.1b).

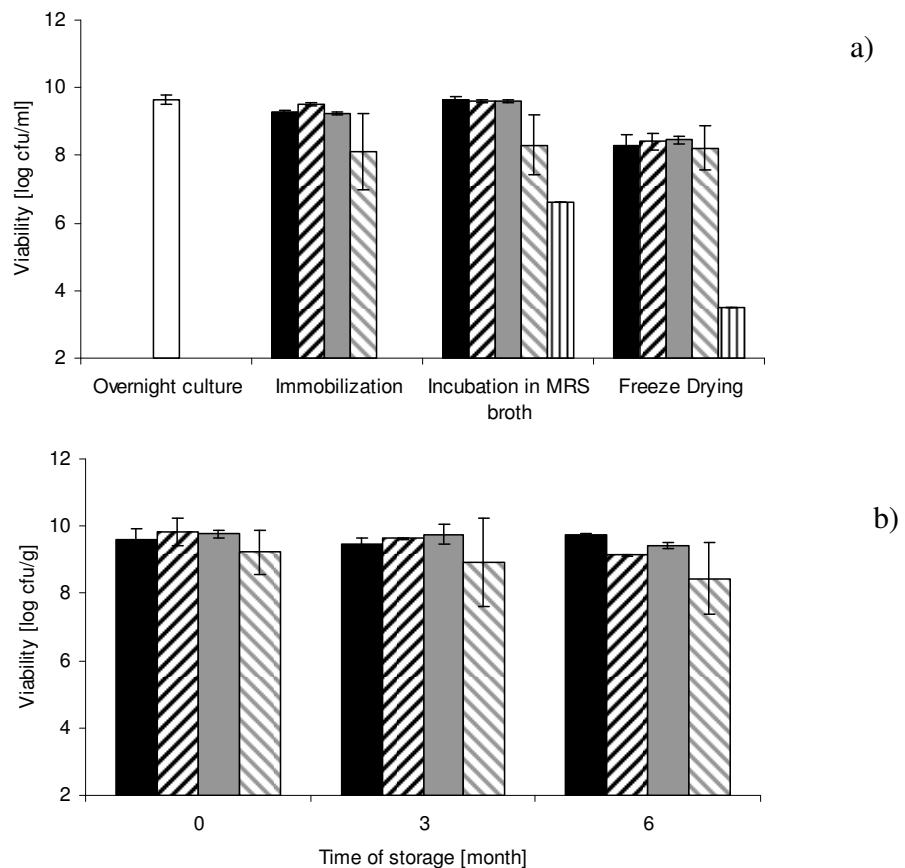


Figure 4.1 Viability of *L. plantarum* (mean and SD, n=3) in calcium alginate films obtained with 0.8M CaCl<sub>2</sub> (black bar), 1.6M CaCl<sub>2</sub> (dashed black bar), 3.2M CaCl<sub>2</sub> (grey bar), 6.4M CaCl<sub>2</sub> (dashed grey bar) and 12.8M CaCl<sub>2</sub> (striped black bar) before and after processing (a) and throughout 6 months of storage at 4°C (b).

Lactobacilli loaded films were characterized by smooth surface morphology, while blank films by rough (Figure 4.2).

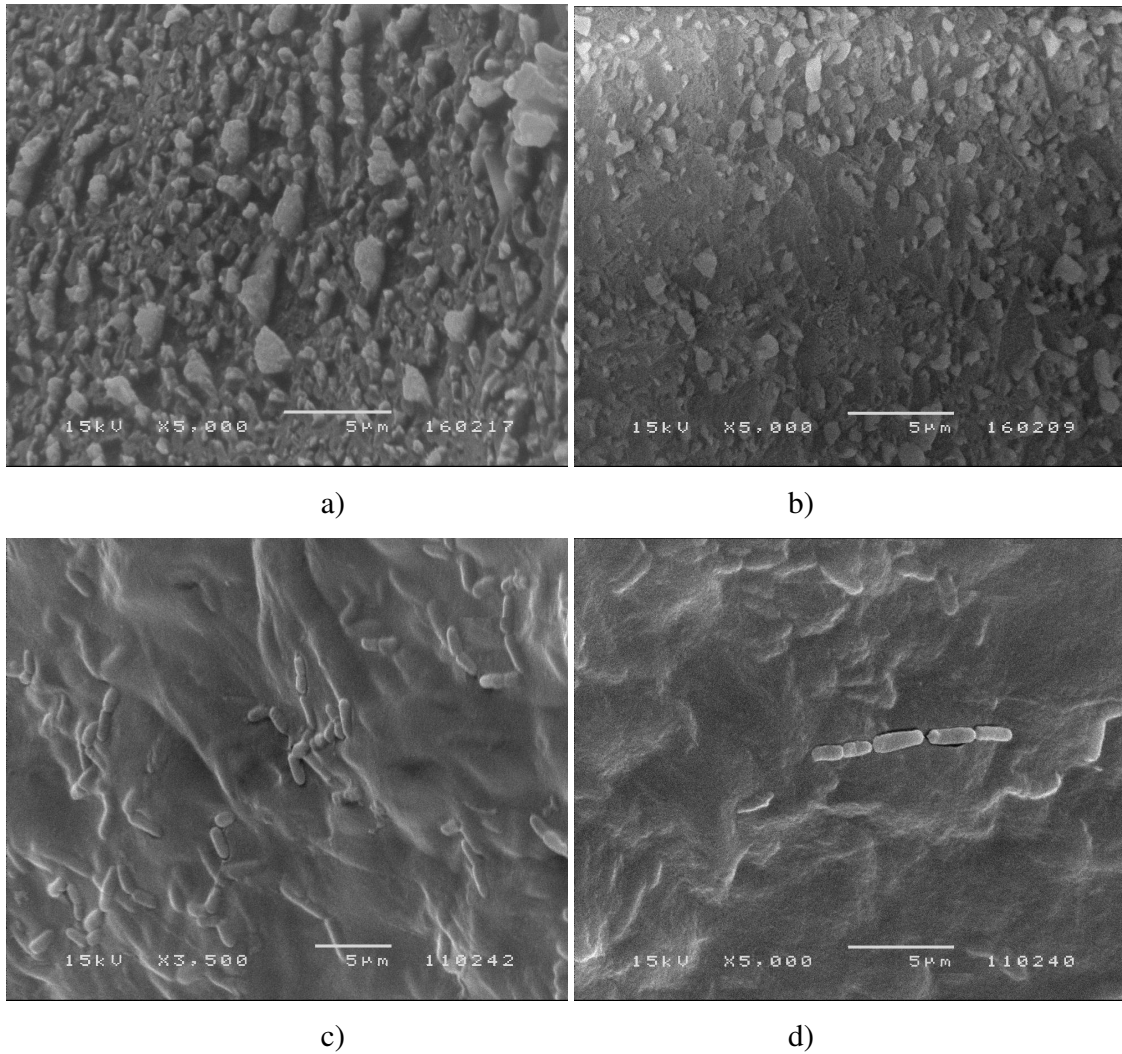


Figure 4.2 Scanning electron photomicrographs of blank a) external and b) internal surface and *L. plantarum* loaded c) external and d) internal surface alginate films produced with 1.6M  $\text{CaCl}_2$ .



The calcium content of films is shown in Table 4.2. It was well below from the amount applied in film formation in both blank and lactobacilli loaded films. The presence of lactobacilli led to a 1.70 up to 1.95 fold decrease of the calcium concentration compared to blank films. The swelling ratio of the lactobacilli loaded films (8.06 to 10.07) was higher than the swelling ratio of blank films (3.62 to 8.96), i.e., the water absorption capacity increased with the decrease of calcium content. Tensile strength values of the lactobacilli loaded films ranged from 23 to 42 x 10<sup>3</sup> N/m<sup>2</sup>, whereas blank films showed smaller variations of the tensile strength values (44 to 48 x 10<sup>3</sup> N/m<sup>2</sup>). Similarly, Young's modulus values in *L. plantarum* loaded films ranged from 8 to 23 x 10<sup>3</sup> N/m<sup>2</sup> and showed smaller variation in blank films (18 to 24 x 10<sup>3</sup> N/m<sup>2</sup>). Thus, the increase of calcium content in films was reflected by an increase in tensile strength and decrease of elasticity of films. The films bioadhesion values ranged from 50 to 67 x 10<sup>-2</sup> J/m<sup>2</sup>, except of 3.2M blank patch (119 x 10<sup>-2</sup> J/m<sup>2</sup>) (Table 4.2).

Table 4.2 Physical characteristics of calcium alginate films (mean and SD).

	CaCl <sub>2</sub> formation solution [M]	Film calcium content (mM)	Thickness (mm)	Swelling ratio	Crushing force (N)	Tensile strenght (x10 <sup>3</sup> N/m <sup>2</sup> )	Young's modulus (x10 <sup>3</sup> N/m <sup>2</sup> )	Work of bioadhesion (x10 <sup>-2</sup> J/m <sup>2</sup> )	Peak detachment force (x10 <sup>-2</sup> N/m <sup>2</sup> )
Lactobacilli loaded films	0.8	0.91	4.1±0.29	10.07±0.12	0.84±0.05	23±4	8±3	53±5	156±45
	1.6	1.65	4.0±0.12	8.96±0.58	1.09±0.28	35±11	15±2	55±5	145±45
	3.2	2.37	4.0±0.12	8.06±0.62	1.38±0.24	42±7	23±1	56±5	155±38
Blank films	0.8	1.55	3.85±0.11	8.96±0.20	1.33±0.15	44±8	18±1	67±13	87±31
	1.6	3.22	3.84±0.20	7.05±0.61	1.35±0.28	45±4	20±5	50±6	65±11
	3.2	4.55	3.85±0.25	3.62±0.25	1.53±0.25	48±11	24±3	119±28	270±52

The lactobacilli released in the first 10 min from 0.8 and 1.6M films corresponded to 10<sup>8</sup>cfu/g vs. 10<sup>7</sup>cfu/g released from the 3.2M films. In 2 hours the released lactobacilli from the 3 types of films corresponded to 10<sup>9</sup>cfu/g. 0.8M, 1.6M and 3.2M films released their total number of lactobacilli as follows: up to the forth, eight and tenth hour, respectively (Figure 4.3).

The non-processed *L. plantarum* cultures were active against *P. aeruginosa* at a cell concentration of 1 x 10<sup>7</sup>cfu/ml. After the immobilization of *L. plantarum* into alginate freeze-dried films, as well as during storage, the inhibition of *P. aeruginosa* was obtained by 6.2 x 10<sup>7</sup> ± 5.4 x 10<sup>7</sup> *L. plantarum* cells /ml. Inhibition of *P. aeruginosa* was observed

8h after its co-incubation with non-processed or processed to films *L. plantarum* (data not shown).

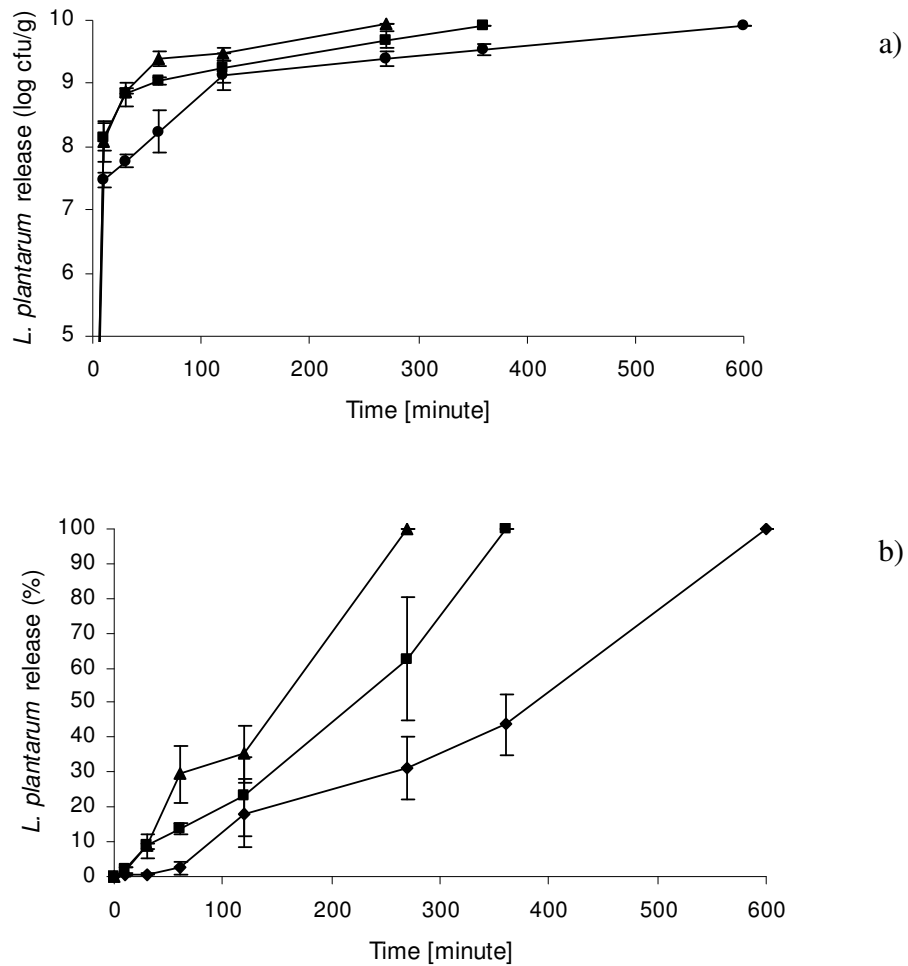


Figure 4.3 Release of lactobacilli from films (formed with ▲ 0.8 M; ■ 1.6M; ● 3.2M CaCl<sub>2</sub>), represented as log cfu/g (a) and in % (b), as a function of time.

#### 4.3.2 In vivo effect of *L. plantarum* films on *P. aeruginosa*

No bacteria were isolated from the liver, spleen, kidney and lungs or from the blood of any rat (groups NBc to BPsL). *P. aeruginosa* and *L. plantarum* were not recovered from blood and organs of groups BL, BPs and BPsL. Various bacterial flora, without *L.*

*plantarum* and *P. aeruginosa*, was found in the intestines of all rats and from the skin of group NBc rats.

The burned wounds of the rats contaminated with *P. aeruginosa* (group BPs) showed microbial counts of  $10^7$ - $10^8$  *P. aeruginosa* / g of skin. The wounds of the rats with *L. plantarum*-films (group BL) showed a bacterial load of  $10^4$  *L. plantarum* / g of skin. In group BPsL, the same number of lactobacilli per gram skin as in group BL was recovered. However, the number of *P. aeruginosa* corresponded to  $10^2$  cells per gram of skin, i.e. a reduction of 5 to 6 log units was observed in the presence of *L. plantarum* loaded films (Table 4.3).

Table 4.3 Microbial counts (mean  $\pm$  SD) in tissue obtained from burn wounds after 8h from group BL (treated with *L. plantarum* loaded films), BPs (contaminated with *P. aeruginosa*) and BPsL (contaminated with *P. aeruginosa* and treated with *L. plantarum* loaded films).

Group	<i>L. plantarum</i> cfu/g skin	<i>P. aeruginosa</i> cfu/g skin
BL	$2.04 \times 10^4 \pm 1.75 \times 10^4$	
BPs		$7.36 \times 10^7 \pm 1.52 \times 10^7$
BPsL	$3.63 \times 10^4 \pm 2.47 \times 10^4$	$3.71 \times 10^2 \pm 2.87 \times 10^2$

To monitor the effect of burn and the presence of *P. aeruginosa* and *L. plantarum* in burn wounds on the distant organ injury in rat, several biochemical parameters were analysed. Blood urea and creatinine concentrations were studied to assess the renal function, while AST and ALT were determined to evaluate the hepatic function (Figure 4.4). Urea and creatinine levels as well as AST and ALT were found to be significantly higher in burned animals (groups Bc to BPsL) than in non-burned animals (group NBc) (Figure 4.4,  $p < 0.05$  to  $p < 0.001$ ). The presence of bacteria did not affect the levels of urea in burned animals. Interesting to point out was the fact that lactobacilli showed a tendency to decrease the elevated creatinine levels induced by burn (groups BL vs Bc). However, when burns were contaminated with *P. aeruginosa* (groups BPs and BPsL) creatinine levels were significantly higher than in non-contaminated burned animals (group Bc) ( $p < 0.05$ ) and treatment with lactobacilli had no effect for the time of the experiment. The presence of *P. aeruginosa* and *L. plantarum* in group BPsL resulted in significantly higher AST and ALT levels compared to burned control animals (group Bc) ( $p < 0.05$ ).

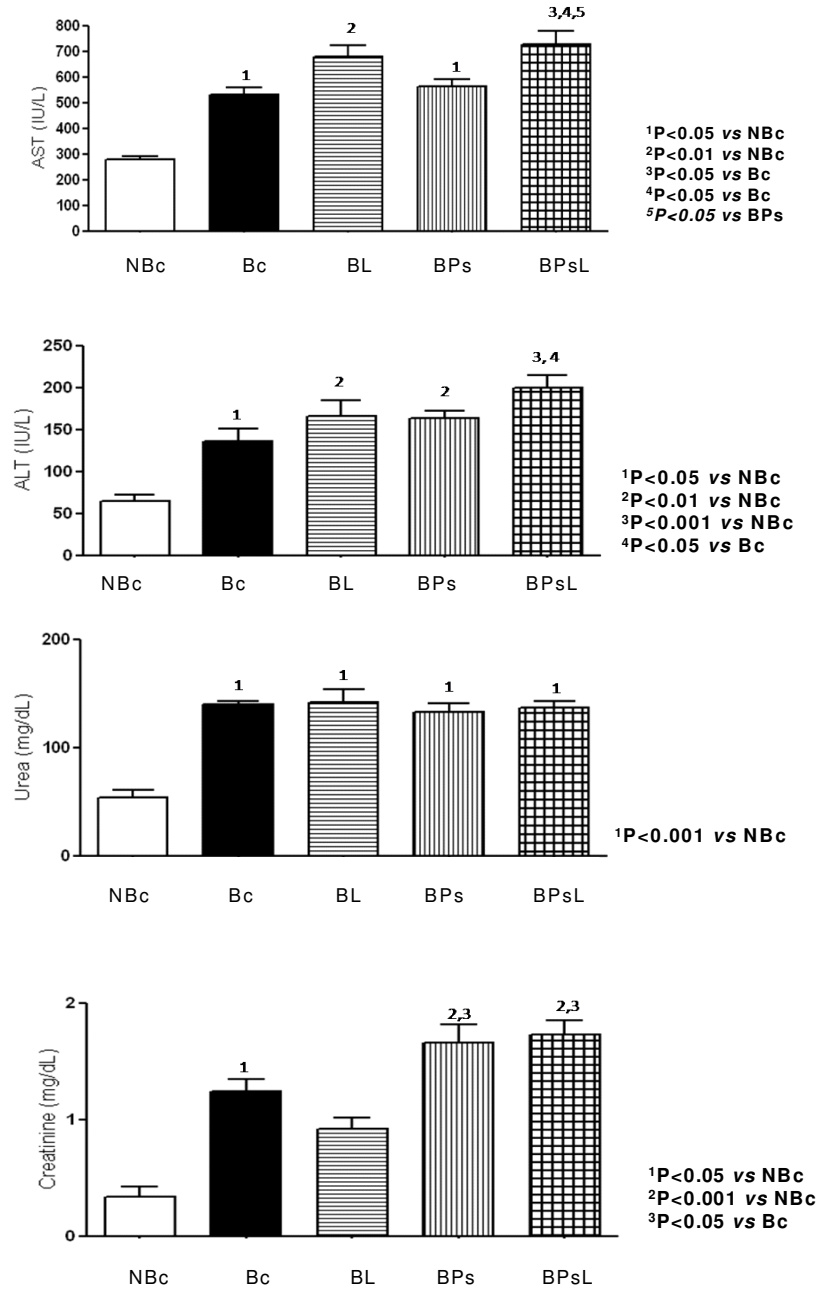


Figure 4.4 Serum aspartate aminotransferase (AST), alanine aminotrasferase (ALT), blood urea and serum creatinine levels in non-burned (NBc) and burned (Bc) control groups and burned and exposed to *L. plantarum* loaded films (BL), *P. aeruginosa* (BPs) and both *P. aeruginosa* and *L. plantarum* loaded films (BPsL) groups.

## 4.4 Discussion

### 4.4.1 Preparation and in vitro evaluation of *L. plantarum* loaded calcium alginate films

When the cell-alginate suspension was added to  $\text{CaCl}_2$  solutions, calcium alginate films were formed. These gel systems evolved slowly with time because of crosslinking reactions, taking place during the gel formation, proceeding progressively from the surface to the interior (Velings and Mestdagh, 1995). The increase of calcium content reduced the time of film formation and allowed the building of a stronger and more compacted network (Table 4.1). The observed decrease in film volume could be due to network formation, characterised by higher complexing between the carboxylate groups of the guluronate monomers of alginate and the calcium cations, resulting in reduced space occupied by alginate and therefore decreased film volume (Velings and Mestdagh, 1995).

Figure 4.1 shows the viability of *L. plantarum* during film production and storage. The amount of calcium added to the film was critical to both film formation and lactobacilli viability. Higher the amount of calcium higher the tensile strength of the film but lower the number of viable bacteria. Figure 4.1a shows that immobilization with high concentrated calcium solutions (6.4 and 12.8M) was reflected by a decrease in the number of bacteria. Intracellular concentration of calcium in bacteria is tightly regulated ranging from 100 to 300nM (Dominguez, 2004). Calcium concentration in bacterial growth media can be approximately 20 $\mu\text{M}$  (Rosch et al., 2008). Generally, extracellular calcium concentration higher than 10mM is considered to be toxic to cells (Wan et al., 2008). In the present study, the millimolar range of calcium measured in the films (Table 4.2), when higher than 4.55mM, resulted in a decreased *L. plantarum* viability. The hypertonic calcium solutions used in the film formation could have provoked plasmolysis with the cytoplasm shrinking and the plasma membrane contracting from the cell wall as a result of internal water loss. Consequently, cells may survive and resume growth if favourable conditions are found, as observed during incubation in MRS broth of films formed with high concentrated calcium solutions (6.4 and 12.8M) (Figure 4.1a) (Korber et al., 1996).

The preservation of the films by freeze-drying has shown a decrease in *L. plantarum* viability of about 1 log unit. It must be pointed out that films formed with calcium concentrations up to 3.2 M have shown the smallest decrease in numbers (1 order of magnitude), whereas for higher calcium contents a significant decrease was observed. Different survival rates in films during freeze-drying might be due to the different initial lactobacilli concentration in films. In general, surviving fraction of the microorganisms increases with the increasing biomass concentration during freeze-drying, and this is attributed to the mutual shielding effect of the microorganisms against the severe conditions of the external medium (Bozoglu et al., 1987). It was observed that in the presence of sucrose as a protectant,  $10^9$ - $10^{10}$  cfu/ml was an optimal initial bacterial cell concentration resulting in the highest survivability during freeze-drying (Costa et al., 2000; Palmfeldt et al., 2003). On the other hand, if the number of bacteria in films is too low (films produced with 6.4 and especially 12.8M calcium chloride), the cell viability might be lowered through lack of the protective effects generated by mutual contact of the cells due to lack effective networks (Souzu, 2004). Furthermore, cellular damage induced by the hypertonic calcium solutions could have continued through the freeze-drying procedure and in storage (Souzu, 2004).

Once immobilization is carried out in the best conditions, films were stable overtime (Figure 4.1b). It means that the process of drying was adequate to preserve the bacteria and the calcium alginate with nutrients from the MRS broth left after incubation provided a proper protection to the lactobacilli.

The morphology of the films with and without lactobacilli is quite different. Figure 4.2 shows the surfaces of blank and loaded films. It might be due to the interaction between the lactobacilli cells and the calcium alginate matrix. The rough internal and external surfaces of films were transformed into smooth surfaces during the proliferation of lactobacilli in calcium alginate films. The proliferation of lactobacilli resulted in a decrease of the calcium content in the films, which could be due to production of lactic acid by the bacteria during incubation of films in MRS broth, which competes with the alginate for calcium (Morin et al 1992).

Measurement of calcium content in the films has shown that a large part of calcium was not used in film formation (amount applied *versus* amount quantified). A possible

explanation could be that the upper layers of the films (the first to be formed) prevented calcium to go underneath. Velings and Mestdagh, [12] 1995 described a parabolic distribution in the calcium concentration in the calcium alginate gels, which is higher on the surface of the gel that is in contact with the formation solution than in the centre of the gels.

A decrease in calcium has a destabilizing effect on the network and consequently on the physical properties of the films (Table 4.2). For the same surfaces (3.2 x 2.2 cm) the film strips have shown different thicknesses showing clearly an increase in thickness when lactobacilli are present. This suggests that the network is not so compact, as for the blanks, derived from lower calcium content, as discussed. The smaller compaction as a consequence increases the absorption of water by the films: the blank films show a larger range of swelling ratio (3.62 up to 8.96) than loaded films (8.06 up to 10.07). Results of the force at crush and the tensile strength emphasize the effect of the presence of lactobacilli. Whereas the blank strips show closer range of results (the standard deviations show that they were not significantly different), for the loaded strips the largest tensile strength almost doubles the smallest one. Still with the mechanical properties of strips, the Young's modulus shows that blank strips are less elastic than the loaded ones. This suggests that the network formed by the calcium alginate was stronger and less elastic in the absence of lactobacilli. The calcium content did also affect the release of lactobacilli from films. Increased crosslinking density with calcium and the consequent decrease of porosity (Wan et al., 2008) resulted in slower release of bacteria (Figure 4.3). The lactobacilli were at both the surface of strips and within the network (Figure 4.2 c, d). The presence of surface lactobacilli explained the observed burst effect: 0.3 to 2.5% of the total bacterial load was released immediately after contact with PBS.

The adhesion between the loaded film and chicken skin was reflected by the work of adhesion which was quite constant for loaded strips whereas for blank strips it varied considerably. The explanation can be formed at the surface of the strips. Figure 4.2 clearly shows differences at the surface of strips: loaded strips have a smooth surface covered with bacteria whereas the blank strips have rough surface. The asperities observed might have increased the surface of contact between the strip and the chicken's skin in a non anticipating pattern. The force at detachment showed that loaded strips

required generally a larger force than blank strips. However, the work of adhesion was smaller for loaded strips. It shows that bacteria promoted an easier detachment of films under tension, decreasing the adhesion work but still holding the strip, showing a higher force at detachment.

#### **4.4.2 In vitro and in vivo effect of *L. plantarum* films on *P. aeruginosa***

The inhibitory effect of *L. plantarum* ATCC 8014 against a VIM-2-metallo- $\beta$ -lactamase producing *P. aeruginosa* FFUL 1396 was investigated both *in vitro* and *in vivo*. *In vitro* results have shown that *L. plantarum* cultures at high cell concentrations of  $10^7$ - $10^8$ cfu/ml before and after immobilization into calcium alginate films were effective against the multi-resistant *P. aeruginosa*. Microbial counts of *P. aeruginosa* in skin have shown that lactobacilli have caused a pronounced decrease (5 to 6 log units) of *P. aeruginosa* after 8h in a burn model of rat. These results confirmed an earlier report of the inhibition of a standard clinical isolate of *P. aeruginosa* PA100 by *L. plantarum* ATCC 10241 (Valdez et al., 2005) and furthermore showed that alginate films could be a suitable matrix preserving both viability and antibacterial activity of lactobacilli. In the present study a model of burn injury in rat was used, whereby a group of non-burned rats was compared to burned and contaminated burned rats. The observed increases of transaminases (AST and ALT) levels induced by burn were further elevated by the contamination of the burns with bacteria (group BPsL), suggesting distant organ injury of liver tissues.

Bacterial translocation from the burn wounds was not observed since no bacteria were recovered from the blood, liver, spleen, kidneys and lungs. This observation confirms the results of Peral et al., 2009, indicating that 48h after the end of the treatment of burned patients with *L. plantarum* cultures, this bacterium was not recovered from either peripheral blood or wound samples. This is a promising outcome because in situations where lactobacilli are to be used, the effect occurs at the burned location only.



#### **4.5 Conclusions**

The study confirmed the inhibitory activity of immobilized lactobacilli in a calcium alginate films at a concentration of  $10^8$ cfu/ml in a model of burn injury in the rat.

Films could be considered solid dosage forms which are easier to handle and deliver to patients than the starting cell cultures or other liquid dosage forms. Thus, the developed *L. plantarum* loaded films might contribute to the use of lactobacilli as an alternative in topic burn wound prophylaxis and treatment.

## **CHAPTER 5**

### **CONCLUDING REMARKS**

The present work has confirmed the possibility of using solid dosage forms (tablets, pellets, beads and films) to deliver viable and antibacterially active lactobacilli, as proven by in vitro and in vivo tests.

The factors affecting the viability of the bacteria throughout the manufacture of solid dosage forms were: probiotic strain, formulation into which bacteria were inoculated, pressure applied in processing and process of drying.

As anticipated the spores (*Bacillus subtilis*) were more resistant than vegetative forms (*Lactobacillus plantarum*, *Lactobacillus rhamnosus* strain GG, *Lactobacillus lactis* and *Lactobacillus bulgaricus*, by decreasing order of viability) to processing.

The stabilization of the bacteria has shown that protective matrix (e.g. skim milk) and cryoprotectors (e.g. glycerol) must be present at early stages of bacterial de-hydration by freeze-drying (FD). The choice of excipients is critical for bacterial survival. For instance the presence of skim milk and inulin preserved the viability of bacteria, whereas, microcrystalline cellulose, a common excipient for solid dosage forms, have shown a deleterious effect on bacterial viability.

Pressure, whether applied during extrusion or compaction reduced bacterial viable counts, although a relationship between the pressure applied and the decrease on viability was not clear. Further, pressure applied to dry bacteria (tableting of freeze dried bacteria by direct compression) was preferable to pressure applied to powder mixtures containing fresh bacterial suspensions (extrusion and spheronisation). This emphasizes that freeze-drying was less deleterious to bacteria than air drying. The application of a coat to dried bacteria (pellets and tablets) did not modify their viability.

Contrary to extrusion and compaction, the mild conditions during immobilization of bacteria into alginate beads and films preserved high bacterial viability. Alginate matrix was also suitable for preservation of lactobacilli antibacterial activity.

Finally, the high numbers of viable and antibacterially active lactobacilli present at the end of processing and storage of different dosage forms anticipate the successful use of these systems in clinical applications, concretely in burn wounds.

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