Survival of Bacteria in Pellets, Tablets and Capsules

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ABSTRACT

The survival of probiotic and model organisms in pellets, tablets and capsules was investigated in an attempt to formulate stable solid oral dosage forms, containing probiotic bacteria. Two new incorporation methods were investigated: direct incorporation of bacterial suspensions into pellet formulations and the freeze drying of mixtures of bacterial suspensions with tableting excipients. The mixing of freeze dried bacteria with tableting excipients was also investigated. Gram-negative aerobic (Escherichia coli), Gram-positive aerobic (Staphylococcus saprophyticus and Bacillus subtilis) and Gram-positive anaerobic (Bifidobacterium longum and Lactobacillus acidophilus) vegetative bacteria, together with spores of Bacillus subtilis, were introduced separately into a formulation, which was extruded, spheronised and dried to produce pellets. Spores survived all stages of the process. Survival levels of the Gram-positive organisms after extrusion, spheronisation and drying were significantly higher than the Gram-negative E. coli. The effects of extrusion speed, extrusion die length to radius ratio, and extrusion pressure on the viability of the more sensitive E. coli were investigated. The level of killing was not affected by extrusion speed or die length to radius ratio. However, survival was inversely proportional to extrusion pressure over the range 1-8000 kPa.

A range of compaction forces was employed to investigate the susceptibility of L. acidophilus, incorporated into a lactose and a microcrystalline formulation mix, to the forces produced by tableting. Samples from both mixtures were compressed at pressure ranging from 1-300 MPa. A strong negative correlation between bacterial survival and compaction pressure was observed, suggesting that survival decreased with increase in tablet compaction pressure. Mechanical strength and friability tests showed that all tablets produced were of acceptable strength. Results show that L. acidophilus can be successfully inoculated into a tablet formulation providing the compaction pressure is monitored carefully.

Stability testing of the L. acidophilus formulations showed that freeze dried L. acidophilus does not remain viable after eight and nine days in the mixtures with microcrystalline cellulose and lactose respectively. Capsule filling with the L. acidophilus/lactose mixture was proved to be the most successful approach, since the lethal effects of drying and pressure were kept to a minimum. Furthermore, these capsules were successfully coated at room temperature with an ethylcellulose/amylose colon-specific coat, without loss of bacteria viability. Five percent coat thickness was shown to withstand simulated gastric and intestinal conditions, whilst remaining susceptible to degradation in the colon by bacterial enzymes.
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INTRODUCTION
1.1 Intestinal flora

The foetus in utero is sterile. At birth it becomes contaminated with a collection of microorganisms from the birth canal and the immediate environment. Some of these are potentially pathogenic, and even under hygienic hospital conditions of delivery, there is transfer of faecal bacteria from the mother to the baby (Tannock et al 1990). If contact with the mother is restricted by caesarian delivery and the foetus is maintained in an incubator, the incidence of faecal bacteria, in the newborn’s gut is measurably reduced (Hall et al 1990).

Within days, many of the potentially pathogenic organisms are eliminated by successive changes in the gastrointestinal flora. Lactic acid bacteria and coliforms become the predominant microorganisms in neonatal human and animal gastrointestinal tracts. During weaning the flora again changes dramatically, with anaerobic bacteria predominating (Berg 1996). The intestinal flora of the adult consists of communities of microorganisms that are remarkably stable, even after drastic dietary changes (Drasar and Hill 1974).

Bacteria are not randomly distributed throughout the gastrointestinal tract of adults, but are found in characteristic population levels at particular regions of the tract (Fig. 1.1). A flora of approximately 200 species exists in the oral cavity (Hardie and Bowden 1974). Saliva contains $10^9$ bacteria per ml shed from oral surfaces, such as the tongue and cheeks, whereas dental plaque contains a very dense population of $10^{11}$ bacteria per gram (Hardie and Bowden 1974).

The human stomach and the upper two thirds of the small intestine (i.e. the duodenum and the jejunum) contain only low numbers of microorganisms. Only $10^3$-$10^4$ bacteria are found per ml of gastric or intestinal contents (Tannock 1995). Microbial populations are restricted in these areas because of the low pH (1-3.5) of the stomach contents (Gruber et al 1987) and the swift flow through the stomach and the small intestine due to peristalsis. The principal microbial types in the small intestine are acid-tolerant lactobacilli and streptococci, which unlike the majority of the organisms found in food, survive passage through the stomach (Tannock 1995).

*Helicobacter pylori*, a spiral shaped, highly motile bacterium (Holt et al 1994), is present
Fig. 1.1 The human gastrointestinal tract: alimentary canal and accessory organs.
in the stomachs of a third to a half of the adult population of the world (Blaser 1993). Colonisation of the stomach by *H. pylori* does not produce disease in most people. However, its presence in some individuals has been linked with gastritis, gastric or duodenal ulcers and the development of gastric cancer (Blaser 1987).

The distal small intestine (ileum) is considered as "transition zone" between the relatively sparse flora of the upper bowel and the tremendously high numbers found in the large intestine (Tannock 1995). A more diverse flora with populations up to $10^8$ bacteria per ml is found in the distal small intestine, where peristalsis is decreased and the pH rises to 5 (Phillips 1993).

The large intestine is the primary site of microbial colonisation, probably because of slow intestinal motility and the higher pH of 5, compared to the pH of 1-3.5 of the stomach (Tannock 1995). Approximately $10^{10}$-$10^{11}$ bacteria are found per ml of intestinal contents, comprising an estimated 400-500 species (Moore 1974). It should be emphasized that 99.9% of the intestinal flora are anaerobic bacteria (Berg 1996). The predominant species of the intestinal flora are members of the *Bacteroidaceae*. The second most prevalent class of microorganisms are species of lactobacilli. Third are members of the *Peptococaceae*, including *Ruminicoccus*, *Coprococcus*, *Peptostreptococcus* and *Peptococcus*. Fourth are bifidobacteria. Other species include *Clostridia*, *Megaspharae*, and *Veillonellae* (Mitsuoka 1982).

The terms "normal" or "indigenous" flora are usually used to describe the collection of microorganisms that normally inhabit the gastrointestinal tract and are present in all communities of a particular animal species (Savage 1977). "Transient" flora is a term first used by Savage (1977) to describe organisms not necessarily present in all communities or not even present in all members of a single community of animals. Dubos et al (1964) related the relationship of the indigenous intestinal flora of a given animal species and the animal host to an ecosystem. Any transient species derived from food, water or even another part of the gastrointestinal tract or skin will not establish and instead will pass through the intestine (Berg 1996). Thus a particular microbial species might be indigenous to one region of the gastrointestinal tract but be only transient in another. These ecological principles explain why the population levels and species
composition of the indigenous gastrointestinal tract flora of a particular host remain remarkably constant (Drasar 1974).

1.2 **Infection control by intestinal flora**

It is increasingly accepted that bacteria of the intestinal flora effectively control the growth of potentially pathogenic microorganisms in the gastrointestinal tract of healthy individuals (O'Sullivan *et al* 1992).

Evidence for the protective effects of the intestinal flora comes from various sources. Firstly it can be shown that germ free animals are more susceptible to disease than their conventional counterparts carrying a complete gut flora. This has been shown in mice by infections caused by *Salmonella enteritidis* (Collins *et al* 1978) and *Clostridium botulinum* (Moberg and Sugiyama 1979). For obvious ethical reasons this sort of comparison cannot be made in humans, but it is reasonable to assume they would apply in man.

Another source of evidence that supports the protective effect of the gut flora is the finding that antibiotic-treated animals, including humans, become more susceptible to disease. Pseudomembranous colitis, caused by *Clostridium difficile* is almost always a consequence of antibiotic treatment (Bartlett and Et 1978).

The third source of supporting evidence comes from experiments in which dosing with faecal suspensions prevents infection. For example *C. difficile* infection can be reversed by administering faecal enemas derived from a healthy human adult (Eiseman *et al* 1958, Schwan *et al* 1984). In chickens it has long been known that dosing newly hatched chickens orally with faecal suspension from adult hens prevents the establishment of salmonellae in the gut (Nurmi and Rantala 1973).

1.2.1 **Antibiotic treatment and antibiotic associated diarrhoea (AAD)**

For the past fifty years, the launching of successive highly active antibacterial agents has encouraged their widespread use in daily clinical practice. However, antibiotics administered to treat infections act not only on the infecting species, but also affect the normal bacterial flora (Kager *et al* 1981, Mulligan *et al* 1982, Bodey *et al* 1983, Kager...

Bifidobacteria are the most sensitive species of the intestinal flora to antibiotic treatment. Nakaya et al (1982) subjected beagles, rabbits and humans to intravenous therapy with cephalosporins (cefazolin and cefoxitin) and penicillins (ampicillin and piperacillin) for periods of up to two weeks. They found bifidobacteria species to be the most sensitive to antibiotic treatment. They also noted that changes in bacterial microflora took place within 24 hours of antibiotic administration, and that 3 to 4 weeks were usually required for the recovery of flora to pre-antibiotic levels.

Sakata et al (1986) investigated the influence of antibiotics on the faecal flora of 54 children treated with antibiotics for a variety of conditions (pneumonia, pharyngitis, otitis media or urinary tract infections). A reduction in count of bifidobacteria of between 2 and 4 log cycles and a one log cycle reduction of the lactobacilli count was observed after administration of ampicillin, penicillin V or methicillin. Erythromycin and cefaclor reduced the number of bifidobacteria by one log cycle, but had no effect on the lactobacilli count. Gentamicin produced a 3 log reduction in the bifidobacterial population and a 4 log reduction in the lactobacilli and E. coli populations. Cefpiramid, suppressed normal flora so markedly that almost all species were eradicated and the active growth of yeasts was promoted. Similar reductions were produced by either oral or intravenous antibiotics administration. However, in contrast with the study of Nakaya et al (1982), bifidobacterial populations recovered after only 3 to 6 days.

Sunakawa et al (1985) conducted studies on the effects of penicillins and cephalosporins on neonates, children and adults. The incidence of pathogenic bacteria was highest in patients with decreased immunity, such as leukemic patients and neonates. This correlated with decreases in the normal intestinal flora. Data on anaerobic bacteria were excluded, due to high inter-patient differences. The incidence of diarrhoea was highest after administration of amoxyccillin, either with or without clavulanic acid.
Antibiotic associated diarrhoea (AAD), which is caused by colonisation of the intestinal tract by pathogenic organisms, results from decreased "colonisation resistance" (van der Waaij et al 1971) due to antibiotic administration. "Colonisation resistance" has been attributed by some authors to the protective effect conferred by the anaerobic component of the normal gut flora (Miller et al 1958, van der Waaij et al 1971, van der Waaij et al 1972a, 1972b Welling et al 1980), while other authors have suggested that facultative anaerobic rods have the major protective effect (Freter et al 1972, Koopman et al 1981, Hentges et al 1983, Hentges et al 1985). It was thought that the anaerobic flora of the digestive tract inhibited the growth of pathogens by producing various antimicrobial substances (Bergeim et al 1941, Bohnhoff et al 1964). However, more recent studies have shown that competition for nutrients is the main factor responsible for such inhibition (van der Waaij et al 1972b, van Furth et al 1989).

1.2.2 Pseudomembranous colitis
The reduction or elimination of lactobacilli renders the host vulnerable to infection (Danziger and Itokazu 1995), resulting in the condition known as pseudomembranous colitis, a side effect usually associated with clindamycin or lincomycin treatment, but which can result from administration of ampicillin, tetracycline or chloramphenicol. Although many antibiotic agents may cause gastrointestinal distress and diarrhoea, pseudomembranous colitis is a specific syndrome caused by an overgrowth of toxigenic strains of C. difficile. Approximately 5% of normal adults carry small numbers of this organism in their faeces (Fekety et al 1993). The onset of severe diarrhoeal symptoms two weeks after starting antimicrobial therapy is characteristic of pseudomembranous colitis. Other common features include crampy abdominal pain, a leukocytosis, and an increased temperature. Management of these patients may be extremely difficult because of excessive protein loss, electrolyte imbalance and peripheral circulatory collapse. The first step in treatment is to discontinue the offending antibiotic. This is followed by administration of an anti-clostridial antibiotic such as vancomycin or metronidazole together with fluid and electrolyte replacement. However, relapses of pseudomembranous colitis are common despite the extreme sensitivity of C. difficile to
antibiotics, the absence of metronidazole or vancomycin-resistant strains, and easily achievable bactericidal concentrations (Chang and Et al 1980, Barlett et al 1980).

### 1.2.3 Nectrotising enterocolitis

Intestinal bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Clostridium difficile*, can cause necrotising enterocolitis (Kraus and Hatzopoulos 1995). This results from the effects of these organisms on injured intestinal mucosa. The neonatal intestinal mucosa has low concentrations of IgA and poor non-immunologic defences, such as decreased concentrations of proteases and gastric acid. Necrotising enterocolitis develops slowly over a period of 24 to 48 hours to an advanced stage of shock, peritonitis, and widespread intestinal necrosis. Approximately 90% to 95% of infants with necrotising enterocolitis have received enteral feeding (Koren et al 1985).

Breast feeding reduces infant morbidity and mortality, even in modern industrialised societies (Beerens et al 1980). Breast milk is important in protecting the human infant against infection. It is antimicrobial due to the presence of lactoferrin, immunoglobulins especially IgA, lipids, lysozyme and white blood cells (Mims et al 1998). Breast milk also develops a predominantly bifidobacterial flora in the infant colon (Beerens 1980). It is believed that the high lactose and low protein and phosphate content, together with the presence of N-acetylglucosamin-containing sugars and the low buffering capacity in breast milk, promote the growth of bifidobacteria. Bifidobacteria produce large quantities of lactic and acetic acids, which maintain the pH of the large intestine at around 5. This helps to prevent proliferation of a number of pathogens such as the strains of *Escherichia coli* that are pathogenic to neonates. To promote a bifidobacterial flora in the infant colon, cow’s milk for artificial infant feeding is treated to become humanised. This process involves the addition of lactose and the reduction of protein and calcium phosphate to promote bifidobacterial growth (Bullen et al 1977).
1.2.4 Traveller's diarrhoea

Diarrhoea is by far the most common medical problem among people travelling to the tropical and subtropical areas of Latin America, parts of the Caribbean such as Haiti and the Dominican Republic, southern Asia, as well as north, east and west Africa (Steffen et al. 1987a). If one takes into account that 20-50% of people crossing international boundaries each year will be affected by this illness (Steffen et al. 1986), it is clear that the threat of diarrhoea and the illness itself represent a great economic expenditure for both the travelling public and the host country. The clinical picture of traveller's diarrhoea is remarkably similar regardless of the geographical region. The illness is usually self-limiting (Kateralis and Farthing 1995). Without specific treatment, it has a mean duration of 4 days with a median of 2 days (Steffen et al. 1987b).

Since the syndrome is most often caused by an infection acquired by ingesting faecally-contaminated food or beverages (DuPont 1995), precautions regarding dietary habits remain the best prophylaxis. However, dietary self-restrictions do not always translate to reduced rates of diarrhoeal illness (Scarpignato and Rampal 1995). Administration of normal inhabitants of the intestinal tract, such as lactobacilli (Black et al. 1989, Oksanen et al. 1990), has been tried with promising results. The underlying mechanism of this approach is the implantation of a favourable protective flora in the intestinal tract, which interferes with colonisation by enteric pathogens (van der Waaij 1982). Antimicrobials remain, however, the most successful form of prophylaxis, being effective in up to 90% of travellers (Scarpignato et al. 1995). However, it is well known that the overuse of antibiotics encourages the emergence of resistant strains, causing a major threat to public health (Abbasi 1998).

The key factor on the management of acute watery traveller’s diarrhoea, particularly in infants and young children, is the restoration of water and electrolyte balance (Scarpignato et al. 1995). This does not reduce the duration of the illness (Farthing 1988) but will limit dehydration. Many patients will require no additional therapy, whereas some will need pharmacologic treatment to shorten the duration of diarrhoea or to relieve the accompanying symptoms like abdominal discomfort, nausea and vomiting. A typical one to three day illness is reduced to approximately one day by trimethoprim-
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1.2.5 Radiation therapy
The various gastrointestinal disturbances, most commonly diarrhoea, that occur during and following radiation therapy have long been suspected to result from disruption of the intestinal flora. For example, Cuzzolin et al (1992) observed a significant decrease in normal intestinal microflora and high counts of the pathogenic Clostridium species, after post-operative radiation therapy of patients with carcinoma of the uterine cervix or endometrium. At the end of the therapy the normal microflora was not able to return to normal values.

1.3 Probiotics
The term ‘probiotic’ has been used in several ways: it was originally used to describe substances produced by one protozoan which stimulated another (Lilly and Stillwell 1965), but was later used to describe animal feed supplements which beneficially affect the host’s gut flora. Parker (1974) defined probiotics as “organisms and substances which contribute to intestinal microbial balance”. Fuller (1989) considered this definition too imprecise since it includes antibiotics. He defined the term probiotic as “a live microbial (food) supplement which beneficially affects the host animal by improving its microbial balance”. Important characteristics for organisms to be used as probiotics are: they should be normal inhabitants of the intestinal tract, should survive passage through the upper digestive tract, should be capable of surviving and growing in the intestine, should produce beneficial effects when in the intestinal tract, and should maintain viability and activity in the carrier food before consumption. The organism should also be non-pathogenic and non-toxic (Kim, 1988).

The strains of lactic acid bacteria used in probiotics are mostly intestinal isolates such as Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium bifidum (Fuller 1991). Lactobacillus is a genus of non-sporing, facultatively anaerobic, Gram-positive rod shaped bacteria whose major fermentation end product is lactic acid. In humans these
organisms are found in the intestine, vagina and mouth, where they form part of the normal flora. They are present in dairy, grain, meat and fish products as well as in fresh water, sewage, beer and in fermenting wine and other food products (Holt et al 1994). Bifidobacteria are anaerobic, Gram-positive, non spore-forming, irregular rod shaped bacteria. They produce lactic acid from glucose fermentation. Formerly categorized in the genus Lactobacillus as Lactobacillus bifidus, they have been the separate genus Bifidobacterium since 1962 (Sharpe et al 1962).

Probiotic bacteria have been shown to control various enteric pathogens such as Salmonella typhimurium (Perdigon et al 1990), Shigella (Nakaya 1984), Clostridium difficile (Corthier et al 1985), Campylobacter jejuni (Antoine et al 1989) and Escherichia coli (Juven et al 1991). They may also provide important protection against urogenital pathogens such as Gardnerella vaginalis, Bacteroides bivius, Candida albicans and Chlamydia trachomatis (Klebanoff et al 1991, Hillier et al 1992).

After 50 years in which interest in antimicrobial therapy has focused on the use of chemotherapeutics and antibiotics, there is now a renewed interest in infection control through bacteriotherapy (Bengmark 1998). This results from several factors:

1. A recognition that antibiotic therapy has not always been successful. For example, mortality associated with Gram-negative bacteraemia remains at between 20 % and 40 % (Teuber 1995), about the same as that during the pre-antibiotic era (Felty and Keefer 1924).

2. An increasing awareness of the fact that antibiotic treatment deranges the protective flora, and thereby predisposes to later infections.

3. An increasing fear of antibiotic resistant microbial strains, as a result of widespread over-prescription and misuse of antibiotics.

4. A fear that industry can no longer be able to develop effective antibiotics at a sufficient rate to compete with the development of microbial resistance to old antibiotics.

5. A widespread interest in ecological methods.

With these points in mind the World Health Organisation (WHO) recommends “global programmes to reduce the use of antibiotics in animals, plants and fishes, for promoting livestock growth and in human medicine”, and recommends “increased efforts to prevent
disease through increasing immunisation coverage with existing vaccines, and through the development of newer, more effective and safer vaccines. In addition, several older forms of therapy, including bacterial interference, serum therapy and the use of bacteriophages to kill organisms, may be worth reconsidering” (WHO 1994).

1.3.1 Pathogenicity of probiotics

The first and most important consideration in selecting organisms to be used as probiotics is the absence of pathogenicity of the organism. Lactic acid bacteria, i.e. bifidobacteria and lactobacilli, which have been used as probiotics by humans for centuries in the form of dairy foods such as yogurts, milk, sour milk, buttermilk, cheese, fresh cheese, butter and sour cream, are considered to be safe, non-pathogenic organisms. Documented reports of pathogenicity caused by lactobacilli are few and isolated. They include a case of endocarditis ascribed to Lactobacillus plantarum (Axelrod et al 1973), and a case of pneumonia in a CD4 lymphocyte depleted AIDS patient caused by Lactobacillus GG (Rogasi et al 1998). Also, two reports of pathogenicity ascribed to bifidobacteria are reported: a case of sepsis caused by Bifidobacterium longum (Ha et al 1999) and a case of neonatal meningitis caused by Bifidobacterium breve (Nakazawa et al 1996). These reports of infections are very few. They are isolated cases of infections acquired in hospital by immunocompromised patients.

Spores of Bacillus subtilis are available in Italy as a probiotic preparation for the treatment or prevention of intestinal disorders (Oggioni et al 1998). It is difficult to see how this organism can be beneficial. It is certainly not an intestinal organism and being a strict aerobe, it would not be able to grow or metabolise in the gut. The pathogenic potential of B. subtilis is another reason why it should be eliminated from use as a probiotic supplement. Several reports of serious infections caused by B. subtilis to otherwise healthy individuals exist (Kiss et al 1988, Richard et al 1988, Thomas 1991, Velasco et al 1992, Wallet et al 1996, Oggioni et al 1998).
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1.3.2 Probiotic survival during passage through the gastrointestinal tract

For successful oral administration of probiotic bacteria, the organisms must survive passage through the stomach and the small intestine. Preliminary studies conducted by Robins-Browne and Levine (1981) in non-fasting volunteers demonstrated that most of the lactobacilli in Lactinex® granules (Hynson, Westcott & Dunning, Inc., Baltimore, Md.) a preparation containing a mixture of freeze dried *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*, when given in milk, can survive passage through the stomach and remain in the small intestine for up to six hours.

Pochart et al (1992) reported the survival of a *Bifidobacterium* strain (without, unfortunately, specifying the strain) ingested in fermented milk, after passage through the upper gastrointestinal tract. Intake of $10^{10}$ colony-forming-units (cfu) of bifidobacteria in 400 g fermented milk led to an increase in ileal flow, from $10^4$ to $6 \times 10^8$ cfu of bifidobacteria per hour within two hours. Administration of an antibiotic-resistant bifidobacterium strain for eight days produced a mean level of $6.3 \times 10^3$ cfu per gram of faeces (Bouhnik et al 1992). The authors concluded that bifidobacteria in fermented milk can survive transit through the stomach and small intestine in healthy adults. When ingestion stopped, there was a gradual decrease in number of detected cells, suggesting the exogenously administered *Bifidobacterium* species did not colonize the human colon.

In a study by Conway et al (1987), the ability of different strains of lactobacilli to survive in gastric juice was tested in healthy subjects. Three subjects received *Lactobacillus acidophilus* strain N2 and two subjects *L. acidophilus* strain ADH and *Lactobacillus bulgaricus* respectively. Survival was related to pH and the strain of *L. acidophilus*. *L. acidophilus* strain ADH showed the best survival in gastric juice. This strain had also the best adhesion capacity to human ileal cells. The addition of milk to the microbial supplement caused a rise in pH and increased survival times for all lactobacilli in gastric juice.

1.3.3 Mode of action of probiotics

Several mechanisms have been suggested to explain how probiotic supplements exert their beneficial effect (Table 1.1) (Fuller 1989):
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#### Possible modes of action of probiotics

<table>
<thead>
<tr>
<th>Possible modes of action</th>
<th>Details</th>
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<tbody>
<tr>
<td>Suppression of viable count by:</td>
<td>production of antibacterial compounds</td>
</tr>
<tr>
<td></td>
<td>competition for nutrients</td>
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<tr>
<td></td>
<td>competition for adhesive sites</td>
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<tr>
<td>Alteration of microbial metabolism:</td>
<td>increased enzyme activity</td>
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<tr>
<td></td>
<td>decreased enzyme activity</td>
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<tr>
<td>Stimulation of immunity:</td>
<td>increased antibody levels</td>
</tr>
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<td></td>
<td>increased macrophage activity</td>
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Table 1.1 Possible modes of action of probiotics as reviewed by Fuller (1989).

For most of these mechanisms there is support from *in vitro* or *in vivo* experimental data. For example, the suppression of viable counts of pathogens by production of primary metabolites such as organic acids or hydrogen peroxide has been proven *in vitro* (Hentges 1983) and suggested to be the cause of growth inhibition (ten Brick *et al* 1987).

However, although several high molecular weight antibacterial substances have been shown to be produced by lactic acid bacteria (*Larsen et al* 1993, ten Brink *et al* 1994, Hauge *et al* 1998) none are known to be active in the intestine.

Competition for nutrients has been demonstrated *in vitro* using *Escherichia coli* embedded in agar slices (Freter *et al* 1983). After preincubation of these slices in human faeces, to introduce probiotic bacteria, growth of *E. coli* depended on the nutrients which penetrated the agar slices during preincubation. Control experiments demonstrated that both the maximal growth rate (*k*<sub>max</sub>) and the yield of colony forming units of *E. coli* depended on the quantity and the quality of the nutrients available within the agar slices.

Competition for adhesion is another mechanism which has been proposed to prevent colonisation by pathogens. This competitive exclusion effect has been demonstrated in chickens where lactic acid bacteria have been shown to remain attached to the intestinal wall after washing four times in buffered saline (Starvic *et al* 1987). This sort of evidence suggests that it is desirable to use adhering strains when designing probiotic supplements. However, it should be noted that adhesion varies between strains of the same species.
(Barrow et al 1980) and can be influenced by the growth conditions and media used (Fuller et al 1975).

The way that lactobacillus supplements can influence microbial metabolism in the gut is demonstrated by the work of Goldin and Gorbach (1984) who showed that feeding *Lactobacillus acidophilus* to human subjects suppressed the levels of microbially-produced β-glucuronidase, nitroreductase and azoreductase.

Animals with a complete gut flora have higher phagocytic activity and immunoglobulin levels compared to germ-free animals (Bealmear et al 1984). A strain of *Enterobacter faecium* established as a monoassociate in the guts of germ-free mice reduced the levels of *Salmonella typhimurium* in the spleen (Roach and Tannock 1980), implying a systemic immunological effect. Yoghurt has been shown to increase antibody levels when fed to germ-free mice (Wade et al 1984) and *Lactobacillus casei* stimulates phagocytic activity when administered orally to mice (Perdigon et al 1986). *L. casei* and *Lactobacillus plantarum* also stimulate phagocytic activity when given parenterally (Saito et al 1981, Kato et al 1983, Bloksma et al 1981). In order for the bacteria to have these kinds of systemic effects it may be necessary for them to migrate from the gut into the systemic circulation. It has been suggested that lactobacilli can translocate and survive for many days in the spleen, liver and lungs (Bloksma 1981).

### 1.3.4 Efficacy of probiotics

Bacteriotherapy with lactobacilli was reported as early as 1908: “to arrest intestinal purification and to disinfect the intestine” (Metchnikov, 1907). Since then a variety of preparations have been developed for clinical use in humans or animals and a substantial number are under test. Many Bifidobacteria and Lactobacilli preparations exist on the market, classified as food supplements. Clinically, they are considered as a natural and milder alternative to chemical antimicrobials, lacking the contra-indications and side effects of the latter. The indications for these preparations vary from vaginitis (Parent et al 1996), diarrhoea caused by radiation therapy (Mettler et al 1973, Salminen et al 1988) or antibiotic administration (Sanders 1994) to traveller’s diarrhoea (Oksanen et al 1990, Sanders 1994).
The efficacy of these lactic acid bacterial supplements in the treatment of traveller’s or antibiotic-induced diarrhoea is debatable. Sanders (1994) examined the role of lactic acid bacteria as a promoter of health and concluded that “this research area has suffered from a lack of coordinated efforts between the clinicians and the microbiologists, and that differences in strains, levels, model system and stringency of data interpretation lead to apparent inconsistencies in conclusions from published research”.

Pozo-Olano et al (1978) administered 4 Lactinex® tablets at each mealtime for eight days to volunteer travellers to Mexico from the United States in a double blind clinical trial. The volunteers were observed for four weeks, both home and abroad. After comparison to the placebo group, the researchers concluded that prophylactic ingestion of lactobacilli for one week did not reduce the incidence or duration of traveller’s diarrhoea either during the period of ingestion or during the following three weeks.

To evaluate the efficacy of Lactinex® against enterotoxigenic *Escherichia coli*, Clements et al (1981) carried out a double blind randomised clinical study in which 48 volunteers were “challenged” with *E. coli* strains that produced heat-stable or heat-labile enterotoxins, or both. *E. coli* “challenge” was performed by drinking an *E. coli* suspension. The researchers attempted to maximise small bowel colonisation by lactobacilli by administering Lactinex® in milk and in a six hour interval regimen during 36 hours before and 96 hours after *E. coli* “challenge”. No significant differences were observed between the Lactinex® treated and the placebo groups.

Tankanow et al (1990) administered Lactinex® or placebo four times a day for ten days to 38 pediatric patients, of mean age 29 ± 17 months. The patients were receiving concurrent amoxycillin therapy. Lactinex® had no significant effect in preventing diarrhoea in the patient population studied. However, the authors stress that patient’s age, diet and parental definition of diarrhoea were factors that may have influenced the results.

The above studies may have been flawed by imprecision in the aetiology of the resulting diarrhoea. Gotz et al (1979) studied prophylaxis with Lactinex® against ampicillin-associated-diarrhea. Initially, they found no significant statistical difference between patients receiving Lactinex® and the placebo group. However, when diarrhoea was classified according to its most likely aetiology, causes other than ampicillin were
implicated in 50 % of the patients. These causes included the patient’s diet and medication other than ampicillin. Examples were: magnesium hydroxide administration for constipation; neomycin, castor oil and bisacodyl for hepatic encephalopathy; magnesium hydroxide for ulcer treatment and constipation; magnesium hydroxide, bisacodyl and magnesium citrate as preparation prior to barium studies, and excessive consumption of prunes. When these patients were excluded, the researchers found the difference between the Lactinex®-treated group and the placebo group statistically significant with Fisher’s Exact Test.

Promisingly, a lactobacilli strain recently identified as *Lactobacillus rhamnosus* (Lee and Salminen 1995) and previously known as *Lactobacillus casei* strain GG¹, has been shown to exert a positive effect in the prevention and treatment of diarrhoea in premature infants (Millar et al 1993), neonates (Sepp et al 1993), children (Isolauri et al 1995) and travellers (Oksanen et al 1990, Goldin et al 1992). It has also been reported to be effective against severe intestinal infections such as *Clostridium difficile* (Gorbach et al 1988, Biller et al 1995). It has been available in the Finnish market since 1990 and has been shown to be safe when administered to humans (Donohue et al 1996). Some examples of the promising results demonstrated with *L. rhamnosus* are the following trials:

Eight hundred and twenty persons travelling from Finland to two destinations in Turkey were randomised into two groups receiving either freeze dried *L. rhamnosus* or placebo by Oksanen et al (1990). During the trip, the participants were closely followed by Finnish physicians, and symptoms of gastrointestinal infection were recorded. On the return flight, each participant completed a questionnaire on the incidence of diarrhoea and related symptoms during the trip. The total incidence of diarrhoea in travellers to both destinations was 46.5 % in the placebo group and 41.0 % in the *L. rhamnosus* group. Although these differences are not statistically significant, *L. rhamnosus* appeared to effectively reduce the occurrence of traveller’s diarrhoea in one of the two destinations.

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¹ For ease of comprehension the *Lactobacillus* strains named as *Lactobacillus casei*, *Lactobacillus casei* strain GG or *Lactobacillus GG* will be referred to as *Lactobacillus rhamnosus* in the following text.
In another study (Siitonen et al 1990), patients receiving erythromycin plus *L. rhamnosus*-fermented yoghurt developed significantly fewer diarrhoea episodes than those receiving erythromycin plus pasteurised yoghurt. Isolauri et al (1991) showed that when *L. rhamnosus* was given in fermented milk or as a lyophilised powder it shortened the duration of diarrhoea in well nourished Finnish children. Daily administration of *L. rhamnosus* to elderly nursing home residents was also associated with normalisation of loose stools (Ling et al 1992).

Silva et al (1987) suggested that the reasons behind these promising results lie in the ability of the *rhamnosus* strain to adhere to the human epithelial cells, its resistance to bile, its elaboration of antimicrobial factors, and its ability to transiently colonize the human intestine. Growth of this strain also reduces the level of fecal enzymes such as β-glucuronidase (Saxelin et al 1991). Of the above, it is the ability of the *rhamnosus* strain to adhere to epithelial cells which is crucial (Isolauri et al 1993). It had been assumed that if a lactobacillus continued to be excreted in faeces several days after termination of its supply, it was likely to be mucosa-adhesive. However, this is not necessarily so. The only way to prove true mucosal adhesiveness is with repeat studies using colonoscopy-assisted biopsies (Molin et al 1993). A simpler alternative is to study mucosa adherence *in vitro* by using human epithelial cells such as Caco-2 and HT-29 (Chanviere et al 1989, Adlerberth et al 1996). However, the extent to which *in vitro* results correspond with *in vivo* conditions remains to be confirmed.

Chanviere et al (1989) found that common commercial strains such as *L. bulgaricus* and *L. acidophilus*, which are the only probiotics contained in Lactinex®, are not adhesive to Caco-2 cells nor *in vivo* in humans. They also showed the bifidobacterial strains they tested to be either non-adhesive or only slightly adhesive.

Silva et al (1987) and Elo et al (1991) showed that *L. rhamnosus* demonstrates consistent adhesive properties, which are independent of freeze drying. This finding explains the earlier observations by Goldin et al (1992) that *L. rhamnosus* persists in 87 % of faecal samples after four days and in 33 % after seven days. Ling et al (1994) found *L. rhamnosus* in 28 % of faecal samples as late as two weeks after the supply of the strain had been discontinued. Adlerberth et al (1996) using the HT-29 cell line, showed that
several strains of *L. plantarum* display strong, sometimes unique adhesiveness *in vitro*. In sharp contrast with the abundance of studies using lactobacilli, published reports on the efficacy of bifidobacteria as microbial supplements are few. Colombel *et al* (1987) in a double blind, placebo controlled study gave three yogurts per day containing *B. longum* or placebo yogurts to healthy volunteers who were taking erythromycin one gram orally twice a day for two study periods of three days each. It was found that the simultaneous intake of erythromycin with the bifidobacteria-enriched yogurt reduced the frequency of gastrointestinal disorders. In addition, the researchers detected clostridial spores in only one (out of 10) patients taking the bifidobacteria-enriched yogurt, as opposed to seven (out of 10) of those taking placebo.

Mettler *et al* (1973) studied a group of women with colon carcinoma who were treated with radiation. Fifty per cent of patients were supplemented with an oral bifidobacterium supplement (unfortunately the researchers did not specify the *Bifidobacterium* strain). Thirty per cent of the patients treated with bifidobacteria showed diarrhoea, compared with 65 % in the control group.

As noted above, these isolated studies are too few to draw any firm conclusion on the use of bifidobacteria as probiotics. However, the preliminary reports are promising and support the appearance of a number of bifidobacterial nutritional supplements on the UK and international market.

### 1.3.5 Delivery of probiotics

Yoghurt or milk have been used as vehicles for the administration of lactobacilli in clinical trials (Conway *et al* 1987). These are not only readily acceptable by the patient but also by increasing the pH the survival of the lactobacilli will increase. However there are problems limiting the use of yoghurt or milk-based lactobacilli supplements in community settings. These relate to the difficulty of maintaining suitable refrigeration conditions (Sheen *et al* 1995). In addition, lactose present in yoghurt may also present problems for patients with lactose intolerance.

Lactinex®, a widely used lactobacillus supplement presented either as tablets or as granules which are sprinkled on food, has been associated with poor patient compliance.
In a study conducted by Tankanow et al (1990), the most frequent reason for withdrawal from the study was the child's refusal to take Lactinex® due to its gritty and bitter taste. The large number of probiotic supplements on the UK market are not currently regulated by the Medicines Control Agency (MCA). The reason for this is that these products do not make any medicinal claims: they do not claim to "treat, diagnose or prevent disease, or otherwise prevent or interfere with the normal operation of a physiological function permanently or temporarily, nor do they claim to terminate, reduce, postpone, increase or accelerate the operation of any physiological function in any way" (Medicines Act 1968). Thus bacterial supplements are sold to the general public under food law, i.e. their safety has to comply with the Food Safety Act (1990). The implications of the legislation are that as long as the safety of supplements containing probiotic organisms is assured, the stability of probiotic organisms during storage is not obligatory. Supplements, such as Acidophilus® by Health Aid and Lactobacillus® by Solgar state on their packaging the number of viable probiotic bacteria present at the time of manufacture. These supplements do have a shelf life date, although it is not clear whether the date refers to the stability of the probiotic organisms or the date after which the supplements are not safe to consume.

These minimal legislative requirements have resulted in poor quality preparations. Clements et al (1983) found that two batches of the same product gave different results when used to treat experimentally induced Escherichia coli diarrhoea in human adults. Some preparations claiming to have viable cells present in large numbers have been shown to contain only very low numbers and others which claim to have one species of microorganism contain a totally different species (Fowler 1969, Gilliland 1981).

Some progress has been achieved in the field of probiotics. We now know that the ability of a probiotic strain to adhere to the intestinal mucosa is the key to efficacy. However, if probiotics are to be used as medicines and are under control of the Medicines Act, the stability of the probiotic organism(s) over the shelf life of the product must be demonstrated. There is an absence of good data examining stability of probiotics in supplements whether as powders or in tablets, capsules or granules. Clearly, there is a need for more studies investigating the delivery of these microbes, particularly in tablets.
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and capsules. If such solid dosage forms could be produced successfully, this would not only improve the accuracy of dosing, but would also improve patient compliance.

1.4 Solid dosage forms

It is clear from the above that if a microbial supplement is to be registered as a Medicinal Product it would be required to deliver high numbers of probiotic bacteria in a uniform dosage. Studies (Okada 1984) have shown that as early as 8 days after termination of bifidobacteria supplement intake, the bifidobacterial populations drop to pre supplement levels. This necessitates continuous dosing with the probiotics formulated in a highly patient acceptable form, such as tablet or capsule. The latter would assist in masking any unpleasant taste or odour originating from the microorganisms.

Solid formulations in the form of capsules has been around for nearly 160 years. There is evidence of handmade capsules being manufactured as early as 1834 (Alpers 1896). William Brockedon (1843) was granted a patent for a machine to press tablets. Pellets are a newer pharmaceutical form (Conine and Hadley 1970), which is beginning to dominate the fields of controlled release medications and coated formulations. Compared to liquids, all the above solid formulations offer high patient acceptance rates, except perhaps in young children where swallowing large tablets or capsules may present difficulties (Armstrong 1988). These solid dosage forms encourage patient compliance not only because of their ability to mask unpleasant tastes or odours, but also because of their portability. They also offer ease of dispensing and handling for the pharmacist. If properly developed, they can offer superior dose uniformity with the ability to pack high bacterial populations into a single unit. For the manufacturer they offer an easily transported and marketed product, with low production costs once the initial capital cost has been of set.

1.4.1 Tablets

The earliest reference to tablets as dosage forms is in a report in the arabic literature, in which drug particles are compressed between the ends of engraved ebony rods with force applied by means of a hammer (Armstrong 1988). Details of the tableting process as we
now know it were first published in 1843, when Thomas Brockedon was granted a patent for "manufacturing pills and medicinal lozenges by causing materials when in state of granulation, dust or powder, to be made into form and solidified by pressure into dies" (Brockedon 1843). Brockedon’s invention was utilized in Nelson’s tablet compresser (Nelson 1897), Remington’s pill press (Kebler 1914), and Rosenthal’s screw press (Foote 1928). However, the breakthrough in tablet production was the invention of the rotary tablet machine (as reviewed in Jones 1983). Today, more than 300 monographs for tablets exist in the British Pharmacopoeia, far more than any other dosage form.

Although the principles of tablet production remain the same as 150 years ago, the methodology is a lot more sophisticated.

The production method and required excipients are selected according to the drug substance, the type of tablet to be produced and the pre-compaction treatment. For example the stability of the drug to moisture or heat will determine whether pre-formulation treatments such as wet granulation are necessary. The compatibility of the active material with the excipients will govern their selection. The drug dose might indicate a need for a bulking agent, or otherwise called filler. The drug solubility together with the proportion of the drug in the tablet might indicate the need for a disintegrant agent (Armstrong 1988).

A traditional method of optimising particle size and flow is a pre-formulation treatment termed wet granulation. In this method a granulating solvent, usually water or rarely an organic solvent such as isopropanol, together with a binder are added to the blend of drug and filler. The wet mass is sieved, dried and any additional size reduction is performed. The dry granules may then be mixed in a second dry blending to introduce additional excipients such as lubricants, glidants, disintegrants, flavours or colourants. However, advances in the excipients and the invention of new excipients have made direct tablet compression increasingly feasible for a wide variety of drugs, thus achieving simpler, faster and cheaper tablet production.

Direct compression is a process by which tablets are compressed directly from powder blends of the active material and suitable excipients which will allow uniform flow into
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a die cavity and form a firm compact. The most widely used diluents that need no prior manipulation are microcrystalline cellulose (Avicel PH), microfine cellulose (Elczema), spray dried lactose, modified starch, dextrose-maltose (Emdex) and dicalcium-phosphate (Emcompress). However, tablets produced by direct compression may have a non uniform drug content more often than those which were produced by wet granulation. This is due to drug and diluent differences in particle size and bulk density resulting in segregation during handling and subsequent unacceptable drug homogeneity.

Tablets are prepared by compressing the formulation mix in a die by the application of forces via two punches. Irrespective of tablet press type the process can be divided into three stages (Armstrong 1988, fig.1.2):

The lower punch falls within the die, leaving a cavity into which particulate material can flow under the influence of gravity.

The upper punch descends, and the punch tip enters the die, confining the particles. Further punch movement applies the compressive force to the particles, which aggregate to form a coherent tablet.

The upper punch withdraws from the die and simultaneously the lower punch rises until its tip becomes level with the top of the die. The tablet is thus ejected from the die and removed from the tablet press.

During the above process a series of events occurs according to the pressures applied (Armstrong 1988, fig.1.3). Under the initial very low forces the particles are rearranged to form a less porous structure. There will be some fragmentation as the rough surfaces move relative to each other and rough points are abraded.

When further movement of the particles is impossible particles begin to fragment and deform. Deformation can either be elastic, which is reversible, or plastic, which is permanent. With too little plastic deformation the particles revert to their shape and cohesion is lost. After sufficient plastic deformation the particles remain deformed after removal of the compression force and cohesion is retained resulting in an intact tablet.
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Die, surface view

Die, section

Position 1
Upper punch is raised; lower punch has dropped

Lower punch

Position 2
Hopper shoe has moved forward over die and granules fall into die

Foot of hopper shoe

Granules

Position 3
Hopper shoe has moved back. Upper punch has come down compressing granules into a tablet

Position 4
Upper punch has moved upwards. Lower punch has moved upwards to eject tablet. The cycle is now repeated.

Fig. 1.2 Movements involved in tablet compression.
Fig. 1.3 Force and displacement data obtained from an instrumented tablet press.
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**Survival of microbes in tablets**

During tablet production care is being taken to protect the product from a gross microbial contamination. Excipients have to comply with the British Pharmacopoeia specification of containing not more than $10^2$ viable microorganisms per gram (as determined by plate count) (British Pharmacopoeia 1998a), machinery is usually cleaned with a disinfectant solution, workers wear protective clothing to reduce microbiological contamination of the product and production takes place in areas where environmental contamination is minimised. These production conditions, although described as “clean” (not more than 300,000 particles per m$^3$ according to the B.S.5295:1976, or not more than 350,000 particles per m$^3$ according to U.S. standard 209b), are far from sterile, and it would be unusual for a tablet to be manufactured aseptically.

A number of reports (Kallings et al 1966a,b, Fisher et al 1968, Jain et al 1978, Somerville 1981, Bos et al 1989) confirm the microbial contamination of tablets. This could lead to deterioration in tablet quality or could cause disease. As a result of these alarming reports, a number of researchers have investigated the factors which affect microorganism survival during tablet compaction. Although their inoculation methods differed, all demonstrated a relationship between compaction pressure and bacterial survival.

Chesworth et al (1977) employed raw materials with naturally high viable counts and quantified the levels of inactivation in both dry blended and wet granulated formulations. Viable counts fell by approximately 66-78% which they attributed to the effects of a combination of heat and shear forces produced during compression. However, Rankell and Higuchi (1968) observed that even if all the compression force was converted to heat then the resultant temperature rise would only be 5°C. In contrast Hanus and King (1968) claimed that the average increase in temperature is highly dependent upon the compression force and the formulation. He measured rises of 15-20°C for compacted calcium carbonate and sodium chloride. Travers et al (1970) measured rises of approximately 15°C for aspirin and sodium chloride and 2°C for the lubricant boric acid. Nevertheless, temperature rises of 15 - 20°C are unlikely during the limited compaction time are unlikely to cause the dramatic decrease (66 - 78%) in the tablet viable count.

Fasihi and Parker (1977) inoculated lactose and acacia with Escherichia coli, Staphylococcus aureus and Aspergillus niger spores and directly compacted the mixtures into tablets. They observed a linear relationship between survival rate and applied pressure. However, such linearity was not observed by Yanagita et al (1978) when compacting crystalline cellulose mixed with freeze dried skimmed milk containing Rhodotorula glutinis, Escherichia coli and spores of Bacillus subtilis. The larger Rhodotorula yeast cells were more sensitive to compaction than the smaller Bacillus subtilis spores over the range of 40 to 60 MNm\(^2\). However, one may argue that this survival was due to the protective spore coat of Bacillus subtilis rather than to size difference. Thick spore coats protect against hostile environments (Hugo and Russell 1998).

In a similar study, Plumpton et al (1982) incorporated dried Saccharomyces cerevisiae, Bacillus megaterium and Aspergillus niger spores by dry mixing into a variety of direct compression excipients and examined the effect of compaction pressure on viability. Survival curves of the microorganisms were non linear, although once again survival decreased with increasing compaction pressure. The level of survival at particular pressures appeared to be dependent upon the size of the contaminating organisms, the ratio of the organism size to particle size of the direct compression excipient and the mechanism of compaction of the excipient.

Fasihi and Parker (1987) prepared lactose tablets, again by direct compression, containing cells of Staphylococcus saprophyticus and spores of Bacillus subtilis. It was found that bacterial survival was dependent on the speed of compaction, the compaction pressure, the degree of densification of compacts and on the compression behaviour of the powders.

In another similar study, Blair et al (1991) grew Staphylococcus aureus and Enterobacter cloacae separately in a suspension containing lactose monohydrate or maize starch or Avicel PH-101, which was later dried and compressed into tablets. Previously Ishag (1973) had demonstrated higher levels of bacterial survival after drying when the bacteria were grown within a suspension of the powder to be tested compared to those which were grown separately in nutrient media and inoculated into the test system. Blair et al (1991)
showed that again, bacterial kill increased with compaction pressure and was related to
the excipient used. They found *E. cloacae* the most sensitive organism which its larger
size is in agreement with the findings of Plumpton *et al* (1982; 1986a,b).

The above studies investigated the factors which govern microbial survival during the
tablet compaction. The only report found in literature of microbial stability after tablet
manufacture is that of Maggi *et al* 1994. They introduced *Lactobacillus gasseri*,
*Bifidobacterium breve*, *Bifidobacterium longum* and *Bifidobacterium adolecsentis* into
vaginal tablets. Two formulations and three suspending fluids were investigated with a
skimmed milk and malt extract producing the highest counts within the tablets. Stability
studies showed that no viable *B. breve* or *B. longum* after three months. However, in the
same formulations approximately 60% of *B. adolecsentis* and *L. gasseri* were still viable.

It is worth noting that no reports of microbial viability after drying of wet granules could
be found. Fluidised bed drying is the most commonly used drying process for wet
granules in the pharmaceutical industry. The temperature of 45° C used in the process,
is lethal to the vegetative cells of most bacteria, fungi and most viruses (Davis and
Dulbecco 1980), therefore minimising suspicions of microbial contamination.

1.4.2 Capsules

The word capsule is derived from the latin “capsula” meaning a small box (Jones 1983).
In pharmacy capsules are edible packages made from gelatin which are filled with
medicines to produce a unit dose, mainly for oral use. There are two types of capsule,
‘hard’ and ‘soft’ gelatin capsules. The hard gelatin capsule consists of two pieces, a cap
and a body that fits inside it. They are produced empty and are filled in a separate
operation. The soft gelatin capsule is manufactured and filled in one operation (Jones
1983).

Capsules are useful containers for solid dosage forms when the active ingredient presents
problems of formulation into pellets or tablets. For example, whereas a poorly
compressible active ingredient makes tablet formulation difficult, a compression stage
is not included in capsule manufacture. This is certainly an advantage in the case of
microbial delivery, because as reviewed in section 1.4.1, compaction is lethal to bacteria.
Especially in the case of two piece capsules, filling with freeze dried bacteria does not present any problems as no granulation is necessary. Also, no drying is required, a stage which may present problems as far as microbial delivery is concerned, due to bacterial killing by heat. It is probably due to the uncomplicated nature of producing two piece capsules containing freeze dried bacteria that an absence of studies investigating capsule filling with bacteria exists. This is further supported by capsules being the preferred form of probiotic supplements on the market.

1.4.3 Pellets

The concept of the multiple unit dosage form was introduced in the early 1950's. These multiple units are referred to as pellets, spherical granules or spheroids. They range in size from 0.5 - 1.5 mm with some as large as 3.0 mm (Gandhi et al 1999). Pellets can be defined as oral dosage forms consisting of a multiplicity of small discrete units, each exhibiting some desired characteristic. Together, these units provide the overall desired dose. Multiple unit dosage forms have numerous advantages over tablets or capsules: Pellets achieve a high degree of dispersion in the digestive tract (Hardy et al 1985), thus minimising the risk of high local drug concentrations, which may damage the intestinal mucosa. A high degree of dispersion also results in maximum drug absorption, reduced peak plasma fluctuations and minimal potential side effects without lowering drug bioavailability (Eskilson 1985). Furthermore, the prevention of high local concentrations of active agent makes "dose dumping" less likely to occur than with reservoir-type single unit formulations (Follonier et al 1992).

Pellets also reduce variations in gastric emptying rates and overall transit times. Thus inter- and intra- subject variability of plasma profiles, which is common with single unit regimes, is minimised (Bechgaard and Hegermann-Nielsen 1978). Although some variability still exists and is dependent on the size and state of the stomach, it is more predictable and less extreme than that encountered with single unit dosage forms (Davis et al 1986 a,b).

For the formulator, compared to single unit dosage forms, pellets have better flow properties, narrow particle size distribution, are a less friable dosage form and make
uniform packing easier (Reynolds 1970; Govender et al 1997).

Pellets offer other advantages to the manufacturer: their low surface area to volume ratio provides an ideal shape for the application of film coating. They can also be made visually attractive because of the various shades of colour that can be applied during manufacture (Ghebre-Sellasie, 1989).

Finally, pellets may be compressed to form tablets or coated to modify release of the active ingredient (Bechard and Leroux 1992). The advantages of using small spherical pellets for controlled release are well documented. Pellets provide a smoother absorption profile from the gastrointestinal tract because pellets pass gradually from the stomach through the pyloric sphincter into the small intestine at a steady rate. Also, pellets can be layered with drug and coated with various polymers to control release rates. Furthermore, different types of pellets with different release rates can be combined in a single capsule to provide the desired controlled release profile (Betageri et al 1995).

The drawback of pellets is that the manufacture of these multiple unit dosage forms is technically more complicated, requiring more time and more expense than that of non-divided forms. It should, however, be noted that their effective marketing often justifies their production cost (Follonier and Doelker 1992).

Pellets can be produced by a variety of methods, the choice depending on their application, the characteristics of their formulation, whether a predominantly spherical shape is essential, and whether production costs impose any limitation.

**Formation from the fluid state**

Droplets produced from the fluid state are predominantly spherical, which is the configuration that has the minimum surface / volume ratio and hence is energetically desirable (Newton 1994). Spherical pellets can be produced from droplets by spray congealing, spray drying, sol-gel transformation where a liquid solution formed into drops is gelled by physical or chemical action, or by controlled crystallisation from a solution (Newton 1994).

In spray congealing a slurry of drug material that is insoluble in a molten mass is spray congealed to obtain discreet particles of the insoluble materials coated with congealed
substances. A critical requirement for this process is that the substances should have a well defined melting point or small melting zone (Ghebre-Sellasie 1989).

In the spray drying process an aqueous solution or suspension of core materials and a hot solution of polymer is atomised into a heated chamber. The water then evaporates and the dry solid is separated in the form of pellets, if the liquid evaporates at a rate faster than the diffusion of the dissolved substances back into the droplet interior, or if due to capillary action dissolved substances migrate out with the liquid to the droplet surface, leaving behind a void (Ghebre-Sellasie 1989, Canefe et al 1997).

**Agglomeration of particles**

This set of production methods is associated with the agglomeration of solids on mixing with liquids. It can be wet spherical agglomeration where the solids are separated from the liquids by a bridging liquid, pellets can grow from powder (melt pelletization), seeds, or smaller pellets using a fluid bed or a mixer / granulator, or fluid bed agglomeration where the liquids are sprayed into a fluidised powder bed (Newton 1994).

Of these fluidised bed technology is used most frequently. During this process, a dry drug form is suspended in a stream of hot air to form a constantly agitated fluidised bed. An amount of binder or granulating fluid is then introduced in a finely dispersed form to cause a momentary reaction prior to vaporisation. This causes the ingredients to react to a limited extent, thereby forming pellets of active components (Gandhi et al 1999).

**Extrusion - Spheronisation**

The pellet preparation technique of extrusion - spheronisation uses mixtures of solids and liquids. Pellets are formed by forming and shaping forces. The process has become widely known since articles were published by Reynolds (1970) and Conine and Hadley (1970). It offers quite a few advantages over the other pellet production processes, namely the production of pellets of a narrow size distribution, which are regular in shape and possess definite surface characteristics. The pellets produced by this method have extremely low friability resulting in few fine particles and little associated waste. The process of manufacture is flexible with a range of sizes of pellets being produced.
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(Reynolds 1970). However, there is a limit to which a drug concentration can be used, due to the minimum amount of excipients that can be used. The drug characteristics can also affect the process e.g. moisture sensitivity will affect the formulation. In some cases though, some drugs still can not be formulated by using this method.

This process pellets production can be summarised in four stages: mixing, extruding, spheronising and drying.

Mixing

The first step of the extrusion spheronisation cycle involves the preparation of the wet mass. During powder mixing the active ingredient is blended with the excipients to prepare a uniform, homogenous mixture prior to wet granulation, which involves the incorporation of the fluid and binders. The most commonly used granulating fluid is water, although in some cases the use of a alcohol or a water - alcohol mixture has also been reported (Vervaet et al 1995).

The amount of fluid is determined by the behaviour of the wet mass during the next stages, the extrusion process (Newton 1994) and the spheronisation process. A formulation too dry will produce reduced sized pellets. However, a formulation too wet or containing water with high mobility will produce pellets which will agglomerate excessively and have an increased size (Fielden 1987).

The formulation mix or otherwise referred to as "the wet mass" by some authors, must process inherent fluidity to allow flow during the process and self-lubricating properties which are essential during the extrusion stage to prevent generation of an excessive amount of heat resulting in premature drying and subsequently poor quality pellets (O'Connor and Schwartz 1989).

Different types of granulators are used to perform the mixing of the powder blend and the granulation liquid. There are three types of processors used to mix different constituents of the powder blend. The most commonly used granulator is a planetary mixer (Robinson and Hollenbeck 1991), although in various cases use of a high shear mixer, sigma blade mixer (Ku et al 1993) and a continuous granulator (Hellen and Yliruusi 1993) has also been reported. However, it is important to note that high shear mixers
introduce a large amount of heat into the mass during granulation, which may cause evaporation of the granulation liquid because of a rise in temperature, thereby influencing the extrusion behaviour of the wet mass. This can be avoided by cooling the granulation bowl (Baert and Remon 1993).

Extrusion

The second step of the process is extrusion, where the wet mass is forced under pressure through a die to form spaghetti-like long rods, commonly termed "extrudate strands". The extrusion process is used not only in the pharmaceutical industry but also in the food, ceramic and polymer industries (Gandhi et al 1999), as well as an alternative method for the manufacture of completely water soluble tablets (Murphy et al 1998).

Various types of extruders are available and their choice depends on the force required during extrusion, the type of die which controls the pellet size, and whether it would be used for industrial or laboratory scale. The characteristics of the end product depend on the rheological properties of the wet mass and the die design and extrusion rate which are properties of the extruder type (Newton 1994).

The types of extrusion devices can be grouped into four main classes: screw, sieve and basket, roll and ram extruders. A screw extruder, as the name implies, utilizes a screw to develop the necessary pressure to force the material to flow through the uniform openings, producing uniform extrudates (Hellen et al 1992).

In the sieve and basket extruders the granulate is fed by a screw or by dry gravity into the extrusion chamber in which a rotating or oscillating device processes the plastic mass through the screen. The basket type extruder is similar to the sieve extruder except that the sieve or screen is part of a vertical, cylindrical wall (Hicks and Freese 1989).

The third class of extruders are the roll extruders and these are also known as ‘pellet mills’ (Gandhi et al 1999). Two types of roll extruders are available (Fielden et al 1992): one extruder is equipped with two contra-rotating wheels, of which one or both are perforated, and the second type of roll extruder has a perforated cylinder that rotates around one or more rollers that discharge the materials to the outside of the cylinder.

The final type of extruder is an experimental device called the ram extruder. The ram
extruder is believed to be the oldest type of extruder and features a piston riding inside a cylinder or channel that is used to compress material and force it through an orifice. The diameter of the die is the factor that controls the diameter of the extrudate, which subsequently controls the mean particle size of the pellets (Conine and Hadley 1970). The extrusion and spheronisation behaviour of wet masses processed by ram and cylinder extruders have been compared by Fielden et al. (1992), who found them to be not always equivalent.

**Spheronisation**

The third step of the process is spheronisation. Spheronisation is a technique of Japanese origin, originally invented in 1964 by Nakahara (1964). However, it wasn’t until 1970 with the publication of the process by Reynolds (1970) and Conine and Hadley (1970) that the technique became widely available. It involves the dumping of the cylindrical extrudates onto the spheroniser’s spinning plate, known as the friction plate. A spheroniser is a device that consists of a vertical hollow cylinder (bowl) with a horizontal rotating disk (friction plate) located inside (fig. 1.4). The friction plate has a grooved surface to increase the frictional forces. Two types of geometry of the grooves exist: the most common is the cross-hatch geometry in which the grooves intersect each other at 90° angles, whereas the other pattern is radial geometry in which grooves emanate from the centre like the spokes of a bicycle wheel. The spheronisation of a product takes 2-10 minutes, and a rotational speed of between 200 - 400 rpm for the friction plate is satisfactory to obtain highly spherical pellets (Vervaet et al. 1995). During spheronisation the cylindrical extrudates are broken by the motion of the spheronisation plate into short cylindrical lengths which are transported centrifugally to the periphery of the plate. The small fragments which are produced agglomerate with the larger fragments and finally the particles are being smoothed, where the short extrudate undergoes a series of shape changes from cylinders with rounded ends to dumb-bells and ellipsoids and finally spheres (Rowe 1985) (fig.1.5). Rowe (1985) attributed these events to frictional forces between the extrudates and the spheronisation plate (fig.1.6a). However, Baert and Remon (1993) suggested that rotational forces as well as frictional are responsible for
Fig. 1.4 Spheroniser featuring cross-hatch geometry plate.
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Fig. 1.5 Shape changes during the spheronisation process.
Fig. 1.6 Pellet forming mechanism according to


(b) Baert and Remon (1993): I. Cylinder, II Rope, III Dumbbell, IV Sphere with a cavity outside, V Sphere.
pellet formation. In this mechanism a twisting of the cylindrical extrudate occurs after
the formation of a cylinder with rounded edges, finally resulting in the breaking of the
cylinder into two distinct parts, both featuring a round and a flat side (fig 1.6b). Because
of the rotational and the frictional forces involved in the spheronisation process, the edges
of the flat side fold together like a flower, forming the cavity observed in certain pellets.
A number of authors have investigated spheronisation variables on the effect of the final
et al 1993, Nesbitt 1994, Sonaglio et al1995) and have concluded that uniformly dense
spherical pellets with narrow size distribution are possible with careful selection of the
formulation, spheronisation speed, load, geometry and diameter of the groves of the
spheronisation plates.

Drying
Finally the wet pellets are dried. The pellets can be dried at room temperature (Fielden
et al 1992), or at an elevated temperature in the fluidised bed dryer (Robinson and
Hollenbeck 1991), in an oven (Goskonda and Upadrashta 1993), in a forced circulation
oven (Govender and Dangor 1997) or in a microwave oven (Bataille 1993). Pellet quality
is dependent on the type of dryer used. According to Bataille (1993), oven drying
provides less porous and harder minigranules and a more homogenous surface than those
dried by a microwave oven. Dyer et al (1994) prepared ibuprofen pellets that were dried
either by tray drying or fluidised bed drying and showed that the choice of drying
technique had an effect on the diametral crushing strength and elasticity of the pellets,
and their in vivo release on the surface characteristics of the pellets. However, Kleinebudde
(1994) showed that freeze-drying prevents shrinking of the finished pellets. The size
distributions of freeze-dried pellets are comparable to those of wet (undried) pellets.
Concerning the formulation of microbial delivery systems, pelletisation by any of the
above methods will impose a variety of physical stresses on the bacteria. The only work
found in the scientific literature investigating the fate of microorganisms during a
pelletisation process is that of Blair (1989). She studied pellet contamination, during
extrusion - spheronisation, using a peptone water suspension of Enterobacter cloacae.
Reduced viability counts of *E. cloacae* after the extrusion and the spheronisation processes were observed. These reduced viability counts were not affected by the ram extrusion speed.

In this thesis, the process of pelletisation by extrusion - spheronisation will be used since it can be performed on a laboratory scale with small batches, and is therefore easily investigated. The physical parameters imposed by the process which are likely to influence the viability of microorganisms include the shearing forces produced during mixing and extrusion, frictional forces produced during spheronisation and dehydration and temperature affects during drying.

### 1.5 Colonic Delivery

An important factor in successful delivery of probiotic microorganisms to the colon will be the protection of the administered organism until it reaches the colon. The journey from the mouth to the colon involves passage through stomach and the small intestine. Both are obstacles that might kill bacteria. The low pH (1-3.5, Gruber *et al* 1987) of the stomach results in a highly acidic environment which is responsible for the very low microbial counts in the stomach (Finegold *et al* 1983). Most bacteria are killed, although *Helicobacter pylori* adheres to the human gastric epithelium (Blaser 1987) causing gastritis and gastric ulcers and may be associated with gastric cancer.

Upon entry into the small intestine, probiotic bacteria will be faced with more obstacles: they have to survive in an environment containing a cocktail of host digestive enzymes. These include trypsin, chymotrypsin, lipase, α-amylase, carboxypeptidase, elastase and phospholipase A. In addition, bile, which is bacteriocidal to many organisms, is secreted by the liver to promote the efficient fast absorption. It is believed that a combination of the many enzymes together with the chemical factors present in bile and the physical factor of peristalsis removes bacteria faster from the duodenum than they can reproduce (Haerberlin *et al* 1993).

However, upon entry into the large intestine or colon, the absence of hostile enzymes and the favourable pH render this site ideal for the establishment of a bacterial flora as described in section 1.1. The pH of about 7.5 in the small intestine is hostile to most
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bacteria. It drops to a more favourable level of 6.37 in the proximal colon, then gradually rises to 6.61 in mid colon and reaches 7.04 in the distal colon (Finegold et al 1983). A lower pH and the absence of digestive enzymes have focused attention on colon specific drug delivery systems during the last decade. Such systems are intended to protect active materials until they reach the colon. In this way, microbes could be delivered to a “sterilised” colon. Two other areas of colon-targeted delivery are currently of great interest.

Firstly, the bypass of low pH in the stomach and digestive enzymes in the small intestine, makes delivery of peptides feasible. Peptides which present an interest for oral delivery include analgesic peptides, contraceptive peptides, oral vaccines, growth-promoting peptides, insulin and vasopressin and proteins such as interferons and blood clotting factors. For the majority of peptide and protein drugs, oral delivery is limited by the following factors (Watts and Illum 1997): degradation in the acidic environment of the stomach, enzymatic degradation in the small intestine and colon, low mucosal permeability, rapid small intestinal transit and extensive first pass metabolism by the absorbing membrane and the liver. It has also been shown that peptides may effectively be absorbed from the colon. Kidron et al (1982) showed that an injection of insulin into the ascending colon caused a 50% decrease in blood glucose levels, whilst the same injection into the ileum had no effects unless it was co-administered with a trypsin inhibitor. The colonic absorption of calcitonin has also been demonstrated in both rats and man (Antonin et al 1992, Hastewell et al 1992).

Secondly, the topical treatment of colonic diseases, such as ulcerative colitis, Crohn’s disease and irritable bowel syndrome could be better treated if site specific delivery of the therapeutic agent was effected. For the treatment of these diseases anti-inflammatory agents, chemotherapeutic agents and / or antibiotics need to be present in the colon. Some drugs in these classes are currently precluded from routine use due to their systemic toxicity. However, site specific local delivery may permit their utilisation (Ashford and Fell 1994). Local delivery to the diseased organ offers many advantages, as outlined by Rubinstein et al (1997) namely a reduced incidence of undesired systemic effects, the ability to cut down on the required dose, supply of drug to the biophase only when it is
required and finally maintenance of the drug in its intact form as close as possible to the target site.

As an alternative to colon specific drug delivery systems, which are delivered orally, one may propose rectal administration. However, this socially unacceptable method of drug administration, fails to deliver the active materials to the required site. Suppositories (Hardy et al 1987a) and enemas do not spread further than the descending colon (Hardy et al 1986).

1.5.1 Colonic delivery systems

The concept of colonic drug delivery is not new (Schanker 1959), although it is only in the last decade that a number of colonic delivery systems have been developed. There have been a number of studies investigating the effect of size of a dosage form on the rate that it moves through the colon. These systems achieve specific, targeted delivery to the colon a number of methods: by pH change, by a timed release colonic delivery system and finally, by enzymic degradation.

pH dependent systems

This method takes advantage of the gradual rise of pH throughout the gastrointestinal tract. These systems use a pH dependent polymer containing carboxyl groups (acrylates) making the delivery system insoluble at low pH and more soluble as pH increases, available commercially as methacryllate polymers (Eudagrit®, Rohm Pharma, Darmstadt, Germany). These polymers, depending on the methylester content are insoluble at pHs below 6-7 but dissolve rapidly upon deprotonation of carboxylic acid groups at pHs above 6-7 (Lehman 1971). In addition, cellulose acetate phthalate has been used to coat capsules containing topically effective beclomethasone dicipionate (Levine et al 1987). Examples of colonic delivery systems using these polymers for colonic delivery systems are illustrated in table 1.2:
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<table>
<thead>
<tr>
<th>Drug</th>
<th>Polymer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ASA</td>
<td>Eudagrit S</td>
<td>SmithKline and French</td>
</tr>
<tr>
<td>5ASA</td>
<td>Eudagrit S</td>
<td>Asacol®</td>
</tr>
<tr>
<td>5ASA</td>
<td>Eudagrit L</td>
<td>Claversal®</td>
</tr>
<tr>
<td>5ASA</td>
<td>Ethylcellulose</td>
<td>Pentasa®</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>Eudagrit S</td>
<td>Thomas et al (1985)</td>
</tr>
<tr>
<td>Insulin</td>
<td>Mixture of Eudagrit RS, L, S</td>
<td>Touitou and Rubinstein (1986)</td>
</tr>
<tr>
<td>Beclomethasone</td>
<td>Cellulose acetate phthalate</td>
<td>Levine et al (1987)</td>
</tr>
<tr>
<td>Budesonide</td>
<td>Methacrylic acid copolymers</td>
<td>Astra Pharmaceuticals</td>
</tr>
</tbody>
</table>

Table 1.2: pH dependent polymers used in colonic drug delivery.

However, there are conflicting reports as to the successful use of pH dependent coatings for delivery to the colon (Hardy et al 1987 b, c) and evidence of clinical failure of 5-aminosalicylic acid coated this way (Dew et al 1982, Rijk et al 1988). The reason for this failure may lie behind the findings of a study conducted by Evans et al (1988), which showed considerable variability in the pH from the ileum to the colon (Table 1.3):

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of subjects</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum</td>
<td>55</td>
<td>6.63 ± 0.53</td>
</tr>
<tr>
<td>Mid small bowel</td>
<td>52</td>
<td>7.41 ± 0.36</td>
</tr>
<tr>
<td>Ileum</td>
<td>58</td>
<td>7.49 ± 0.46</td>
</tr>
<tr>
<td>Right colon</td>
<td>66</td>
<td>6.37 ± 0.58</td>
</tr>
<tr>
<td>Mid colon</td>
<td>51</td>
<td>6.61 ± 0.83</td>
</tr>
<tr>
<td>Left colon</td>
<td>50</td>
<td>7.04 ± 0.67</td>
</tr>
<tr>
<td>Whole small bowel</td>
<td>51</td>
<td>7.30 ± 0.34</td>
</tr>
<tr>
<td>Whole colon</td>
<td>48</td>
<td>6.63 ± 0.67</td>
</tr>
</tbody>
</table>

Table 1.3: Measurements of pH in the lower gastrointestinal tract (Evans et al 1988).
Confirming Evans findings, Ashford et al (1993 b, c) showed that these pH dependent polymers lack site specificity. They studied the stability of Eudagrit S for colonic targeting in vivo and in vitro and found that, although Eudagrit S is capable of protecting a tablet from the hostile environment of the stomach and small intestine, disintegration time and site are extremely variable. They concluded that the pH differences between the small intestine and the colon are not sufficiently large to be exploited clinically. Despite these limitations and problems associated with their use, pH dependent colonic systems have been shown to deliver the majority of an orally administered dose to the colon in a study conducted by Dew et al (1982). Ashford and Fell (1994) argued that the site variability may not be important in certain cases. If, however, the ascending colon is the desired target either for treatment or for its absorption capability then a more site specific agent is required.

**Timed release**

Another group of colonic delivery systems use a time release mechanism, as well as pH sensitive enteric coating. Based on the average time of transit through the small intestine release of the active compound is delayed up to 4 to 6 hours (Davis et al 1986 a, b). Various systems have been described in the literature: Pozzi et al (1994) developed a system called the Time Clock® which uses a hydrophobic material and surfactant coating to delay release.

Niwa et al (1995) developed a four part ethylcellulose capsule: At one end of the capsule body micropores, which allow water permeation, are made. When water enters the capsule, a substance made of hydroxypropyl cellulose swells. When the water insoluble capsule cannot tolerate the swelling pressure, it disintegrates and releases the drug.

Quadros et al (1995) formulated a colonic delivery system relying on a placebo to build in the necessary time delay. This system functions as a typical elementary osmotic pump with the exception that the placebo jacket must first be dissolved and extruded through the delivery orifice prior to dissolution of the drug core (Fig. 1.7).
Fig. 1.7 Colonic delivery system by Quadros et al (1995). The enteric coat protects the system until it reaches the intestine. The placebo mantel must dissolve and extruded through the release orifice until the drug core is released. This builds in the necessary time delay for the system to reach the colon.
A more complicated time delay system is described by Ishino et al (1992). This system comprises of a core tablet containing the active drug and a disintegrant, in a poorly water permeable outer shell. The outer shell delays water permeation for a controlled lag time. Once the outer fluid reaches the inner core tablet, this swells until the outer shell finally disintegrates resulting in rapid drug release. Lag time can be controlled by altering the thickness or the composition of the outer shell.

However, all the above authors have not verified their colonic delivery systems in vivo. In vivo circumstances vary greatly from controlled in vitro studies, resulting in great difficulty to extrapolate results from mechanically simulated intestinal conditions (Ishino et al 1992, Shameem et al 1995). Niwa and Takaya (1995) also suggested that in vivo results obtained from animal models cannot be corelated to humans, because animals have very different gastrointestinal make up. In addition, although the small intestine transit time has been shown to be relatively constant of 3 to 6 hours (Davis et al 1986 a, b), there is still a variation of 3 hours. Hence, a short lag time would result in premature release of drug in the upper regions of the gastrointestinal tract, and a long lag time would not release any drug.

**Utilisation of existing intestinal flora**

These colonic delivery systems utilise the existing intestinal flora, either to convert prodrugs to active compounds, or to perform enzymic degradation of a coated delivery system.

The enzymes produced by the colonic bacteria are capable of a large number of metabolic reactions. However, the two main classes considered reproducible enough to be exploited in drug targeting are the azoreductases (enzymes responsible for reducing azo bonds) and the β-glucosidases (enzymes responsible for digesting a variety of polysaccharides).

Ashford and Fell (1994) indicated that, as with other systems questions concerning the inter-individual variation, the influence of disease state, diet, age, concomitant drug administration (particularly antibiotics), can be raised. There are bound to be individuals or circumstances when any type of delivery system will fail. However, the ubiquitous
presence of bacteria in the colons of individuals, together with the historical evidence of
the consistency of certain species, the successful clinical use of sulphasalazine for over
40 years and the more recent development of olsalazine, both of which rely on bacterial
azo reductases for their action, make this a promising avenue for investigation (Ashford
and Fell 1994).

For many years, the azoreductases have been used to activate prodrugs upon their arrival
to the colon, most notably sulphasalazine. Sulphasalazine is used in the treatment of
inflammatory bowel disease. At least 85 % of sulphasalazine passes unabsorbed into the
colon (Klotz 1985). The molecule comprises of 5-aminosalicylic acid (5-ASA) and a
sulphonamide antibacterial, sulphapyridine joined together by an azo bond (\(-N=\ N-\)). Upon arrival of sulphasalazine into the colon, azoreductases reduce the azo bond to
free 5-ASA (Fig. 1.8), which exerts topical antiinflammatory activity. Sulphapyridine
acts as a carrier to deliver 5-ASA, which is also unabsorbed through the colon. However,
sulphapyridine is well absorbed, giving rise to side effects, and as many as 30 % of
patients are unable to tolerate treatment with sulphasalazine (Peppercorn 1984).
Because of the toxicity of sulphapyridine, there has been as interest in using 5-ASA alone
in the treatment of inflammatory bowel disease. However, 5-ASA is well absorbed from
the small intestine (Jamerot 1989) and delivery via conventional oral dosage forms is
not possible.

Newer generation prodrugs to deliver 5-ASA have been developed. Olsalazine
(Dipentum®) is another dimer of 5-ASA linked through an azo bond (Campbell and
Berlingdh 1988) (Fig. 1.9). This drug is as effective as sulphasalazine in maintaining
remission in inflammatory bowel disease (Sandberg-Gertzen et al 1988) and in treating
mild forms of the disease (Willoughby et al 1988). Other 5-ASA prodrugs described
include balsalazine and ipsalazine (Fig.1.9), in which 5-ASA is azo-linked to 4-
aminobenzoylglycine and p- aminohippurate respectively (Chan et al 1983).
The azoreductases have also been used to break down azo-polymer coatings. The first
work in this field was published in 1986 by Saffaran et al (1986) who described the
synthesis of azo-polymers used to coat gelatin capsules, pellets or simply paper strips
containing insulin and vasopressin. Although the researchers concluded that release of
Fig. 1.8 Bacterial degradation of sulphasalazine. The intestinal microflora reduces the azo bond in sulphasalazine to release 5-aminosalicylic acid (5-ASA) and sulphapyridine. 5-ASA is the active part of sulphasalazine, whereas sulphapyridine is simply the carrier.
Fig. 1.9 **Newer azo-prodrugs.** The intestinal microflora reduces the azo bond in balsalazide, ipsalazide and osalazine to release the active compounds. The dotted line presents the site of bacterial cleavage.
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the drugs was due to bacterial degradation, his results have been questioned (Lloyd et al 1994). Another group of researchers (Lloyd et al 1992) showed that the capsules and the pellets disintegrated as a result of a time-dependent mechanism: the diffusion of water into the capsules resulting in mechanical failure.

A series of hydrogels made by cross-linking copolymerization of $N,N$-dimethylacrylamide, $N$-ter-$t$-butylacrylamide, acrilic acid and 4,4'-di-(methacryloylamino-azobenzene have been described by Brondsted and Kopecek (1992). The various polymers differed from each other by pH-dependent equilibrium degree of swelling, modulus of elasticity and permeability. In addition to their ability to degrade because of the azo cross-linker used, their specificity was increased because of their degree of swelling, which was lower at pH 2 than at pH 7.4. The polymer's degradability in vitro and in vivo was found to be dependent on the degree of swelling of the hydrogels. The higher the degree of swelling, the faster degradation was observed (Brondsted and Kopecek 1992). It appeared that the nature of the azo cross-linker is less important than the swelling characteristics of the azo polymer. Indeed, the role of the azo aromatic chain length of the cross-linker in the degradation of copolymers of 2-hydroxyethyl methacrylate (HEMA) and methylmethacrylate was checked by Van den Mooter et al (1992, 1993) and was found to be of minor importance. The workers concluded that a balance was needed to be achieved between hydrophilicity (provided by the HEMA content) to ensure colonic degradation and hydrophobicity to provide adequate resistance to gastric and intestinal fluid.

Other enzymes which are produced by bacteria in the human colon are $\beta$-glucosidases, which enable bacteria to digest a variety of polysaccharide residues that escape digestion in the upper gastrointestinal tract. Table 1.4 lists substrates that are digested by $\beta$-glucosidases and could be used as coatings for specific delivery to the colon (Macfarlane et al 1992).
Table 1.4: Substrates hydrolysed by glucosidases occurring in the human large intestine (Macfarlane et al 1992).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitobiase</td>
<td>Fungal cell wall components</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>Starch, dextrins, maltose, sucrose</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>lactose, arabinogalactan, mucins, galactomannans</td>
</tr>
<tr>
<td>β-Galacturonidase</td>
<td>Pectin</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>Various plant glucosides (e.g. cyasin, amygdalin, isomaltose, laminarin, β-glucans)</td>
</tr>
<tr>
<td>β-Mannosidase</td>
<td>Galactomannans</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>Various glucuronides (e.g. stilboesterol, benzo(a)pyrine glucuronide), chondroitin sulphate</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cellobiase</td>
<td>Small oligosaccharides, cellulose</td>
</tr>
<tr>
<td>α-Fucosidase</td>
<td>Glycoproteins</td>
</tr>
<tr>
<td>N-Acetyl-α-galactosaminidase</td>
<td>Glycoproteins</td>
</tr>
<tr>
<td>N-Acetyl-β-galactosaminidase</td>
<td>Mucins, bacterial cell wall components</td>
</tr>
<tr>
<td>α-Arabinofuranosidase</td>
<td>Arabinogalactan, xylans</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>Galactomannans, melibiose, stachyose, raffinose</td>
</tr>
<tr>
<td>β-Xylosidase</td>
<td>Xylans</td>
</tr>
</tbody>
</table>

A number of delivery systems based on polysaccharides that are selectively degraded in the colon have been reported. The major attraction of these materials is that they are already approved for use as pharmaceutical excipients (Watts and Illum 1997). However, since that most polysaccharides are hydrophilic and gel forming, methods have to be devised to ensure that drug does not prematurely diffuse from the dosage form before it reaches the colon.

A colonic delivery system digested by β-glucosidases has been developed by Ashford et al (1993a). The system was based on the pectin USP applied as a compression coat to
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tablets and proved capable of protecting the core tablet during conditions mimicking mouth to colon transit. Tablets coated with 700mg of pectin and containing a radiolabelled core were administered to 6 volunteer subjects in a gamma scintigraphy study. All of the tablets disintegrated in the colon although it was unclear whether this was due to bacterial degradation of the pectin or time dependent failure of the dosage form due to diffusion of water into the tablet cores. Further studies indicated that the degree of methoxylation of the pectin and calcium content of the pectin layer could influence the solubility of the layer and its susceptibility to enzymatic degradation (Ashford et al 1994).

Pectin mixed with ethylcellulose has also been used as a tablet coating. A solution of pectin was mixed with an aqueous ethylcellulose preparation (Surelease®) and spray coated onto paracetamol tablets. Depending on the coat composition (the pectin content varied from 40 % to 60 %) and amount applied (20 mg - 32 mg), between approximately 5 % and 30 % of the paracetamol was released after 6 hours at pH 7.4 (Wakerly et al 1996).

A pellet coating comprising of amylose and ethylcellulose has been developed by Milojevic et al (1995; 1996 a, b). The amylose was resistant to pancreatic enzymes but susceptible to degradation by colonic bacteria and the ethylcellulose provided a film with sufficient water resistance. A coating comprising of 1 part amylose and 4 parts ethylcellulose was applied to pellets containing 5-ASA gave prolonged resistance to drug release under in vitro conditions which simulated the stomach and small intestine (Cummings et al 1996). However, release of 5-ASA was rapid when the pellets were incubated in an in vitro colon fermenter model. This coating has also been tested in man. Pellets containing 13C-glucose were coated with the amylose and ethylcellulose mixture and administered to human subjects together with a radiolabelled transit marker (Milojevic et al 1996 b). The appearance of 13CO₂ in breath indicated release of 13C-glucose from the pellets. In the majority of subjects, 13CO₂ did not appear until the pellets reached the cecum. However, the breath measurements indicated that the release of 13C-glucose from the pellets in the colon was slow, indicating slow degradation of the coating.
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The rationale of utilising the existing intestinal flora for colonic delivery is not new. However, the success of older systems together with the development of newer delivery systems, offers endless exciting new possibilities in colonic drug delivery.

1.6 Aim

The major role of the bacterial flora of the large intestine is to protect against establishment of opportunist pathogenic organisms. It’s disturbance or destruction can have serious clinical complications for the host. The efficacy of non-pathogenic bacterial supplements that aim to re-establish the probiotic component of an injured or destroyed bacterial flora has been proven.

Bacteria of the genera *Lactobacillus* and *Bifidobacterium* have been used extensively as probiotic supplements. These supplements are most commonly administered orally for patient acceptability. However, there is a marked absence of reputable and consistent studies investigating product stability and the factors which govern bacterial viability during production and storage of probiotics in oral dosage forms.

This study aims to investigate the effect of physical stresses produced during the preparation of pellets, tablets and capsules. In addition, an amylose coat has been applied to capsules containing probiotic bacteria in an attempt to target bacteria specifically to the colon.
Chapter 2:

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2.1 Materials

2.1.1 Excipients

Avicel PH-101® (Microcrystalline cellulose BP) was obtained from FMC Europe, Brussels, Belgium. It is derived from native cellulose by the acid hydrolysis of wood pulp. It acts as a binding agent and has the ability to absorb large quantities of water and be readily deformable. It is practically insoluble in water, dilute acids, sodium hydroxide, acetone, dehydrated alcohol, toluene and most organic solvents (Mathur 1994).

Lactochem® (Lactose BP) was from Borculo Whey Products, Saltney, UK. It is a white or almost white crystalline powder, freely but slowly soluble in water; i.e. 1 in 5 of water and 1 in 2.6 of boiling water. Lactose acts as a filter in pelletisation to add bulk to the formulation (Goodhart 1994).

Flow Lac®, a spray dried alpha-lactose monohydrate, was obtained from Forum Products Ltd, Surrey, UK. It is a white, fine - milled, crystalline powder, odourless, freely soluble in water and practically insoluble in alcohol. It is used for direct tableting and combines superior flowability with exceptional hardness yield (Forum Products, Technical Brochure).

Ac-Di-Sol® (crocarmelllose sodium) a cross-linked polymer of carboxymethylcellulose sodium, was obtained from FMC Europe, Brussels, Belgium. It is used as a tablet and capsule disintegrant. It is described as an odourless, white coloured powder which is insoluble in water, although carboxymethylcellulose sodium rapidly swells to 4-8 times its original volume on contact with water. Concentrations of up to 5% \(^\text{w/w}\) Ac-Di-Sol may be used as a tablet disintegrant although normally 2% \(^\text{w/w}\) is used in tablets prepared by direct compression and 3% \(^\text{w/w}\) in tablets prepared by a wet granulation process (Weller 1994).

Magnesium stearate BP was obtained from Huls, Milton Keynes, UK. It is a compound of magnesium with a mixture of solid organic acids obtained from fats and consists chiefly of variable proportions of magnesium stearate and magnesium palmitate. It is used as a tablet and capsule lubricant at concentrations between 0.25-5% \(^\text{w/w}\) and also widely used in cosmetics and food formulations. It is described as fine, white, precipitated or milled...
impalpable powder of low bulk density, having a faint characteristic odour and taste. The powder is greasy to touch and rapidly adheres to the skin. It is practically insoluble in ethanol, ethanol 95%, ether and water, whereas it is slightly soluble in warm benzene and warm ethanol 95%. It is hygroscopic and may retard the dissolution of a drug from a solid dosage form; the lowest possible concentration is therefore used in such formulations. There may be variation between batches. The hydrophobic nature of magnesium stearate can vary from batch to batch due to the presence of water soluble surface active impurities such as sodium stearate. Batches containing very low concentrations of these impurities have been shown to retard the dissolution of a drug to a greater extent than when using batches which contain higher levels of impurities (Allen 1994).

**Glyceryl monostearate** was from Huls, Milton Keynes, UK and consisted of not less than 90% of monoglycerides, chiefly glyceryl monostearate (C\(_{21}\)H\(_{42}\)O\(_4\)) and glyceryl monopalmitate (C\(_{19}\)H\(_{38}\)O\(_4\)). It is a white to cream coloured wax like solid in the form of beads, flakes or powder. It is waxy to the touch and has a slight fatty odour and taste. It is used as a nonionic emulsifier, stabiliser, emollient and plasticiser in a variety of food, pharmaceutical and cosmetic preparations. Glyceryl monostearate is used as a lubricant and to sustain the release of active ingredients in tablet formulations (Pagliocca 1994).

**Glucose BP**, also known as Dextrose, was from Merck, Dagenham, UK, and it occurs as odourless sweet tasting, colourless crystals or as a white crystalline or granular powder. It is widely used in solutions to adjust tonicity and as a sweetening agent. Glucose is also used as a direct compression tablet diluent and binder, primarily in chewable tablets. Although comparable as a diluent to lactose, tablets produced with lactose require more lubrication, are less friable and have a tendency to harden. The mildly reducing properties of glucose may be used when tableting to improve the stability of active materials which are sensitive to oxidation. Glucose is also used therapeutically and is the preferred source of carbohydrate in parenteral nutrition regimes (Maclaine and Torr 1994).

**Polyethylene glycol (PEG 4000)**, \(\text{OHCH}_2\text{(CH}_2\text{OCH}_2)_m\text{CH}_2\text{OH}\), where \(m= 69-84\) was of
average molecular weight of 3000 - 4800 and obtained from Hoechst Aktiengesellschaft, Frankfurt, Germany. Polyethylene glycol can be used as a plasticizer, an ointment base, solvent, suppository base, tablet or capsule lubricant, depending on the grade. The USPNFXVII describes PEG as being an addition polymer of ethylene oxide and water. PEG grades 200-600 are liquids, whilst grades 1000 and above are solids at ambient temperatures. Solid grades (PEG ≥ 1000) are white or off white in colour and range consistency from pastes to waxy flakes. They have a faint, sweet odour. PEG 4000 has a viscosity of 110-158 mm²/s and a melting point of 50-58°C (Price 1994).

Amylose is an essential linear α-D-glucan made up of α(1→4)-linked D-glucosyl units. It is a naturally occurring polysaccharide and possesses the ability to form films. For this study amylose extracted from wheat was used. The films are resistant to pancreatic amylase but are degraded by colonic bacterial enzymes (Ring et al 1988). A disadvantage of using amylose in film form is its swelling properties in aqueous media. Control of the swelling is achieved by mixing with polymers (Milojevic et al 1996a), in this study ethylcellulose was used. A solution of amylose-butan-1-ol (5% v/v amylose content), extracted from wheat starch, was supplied from British Sugar Technical Centre, Norwich, U.K. Amylose fractions were prepared by sequential aqueous leaching at 90°C of the starch powder, with nitrogen passing through the slurry to minimise oxidative degradation. Swollen gelatinised granules were removed by centrifugation (2000 rpm) and filtration through a glass sinter filter.

Amylose was precipitated as its butan-1-ol complex. After 24 hours at 1°C, amylose butan-1-ol complex was collected by centrifugation (2000 rpm).

Aqueous amylose forms a relatively stable complex with butan-1-ol such that amylose assumes a helical conformation. On heating during the coating process, the complex melts and the butan-1-ol evaporates leaving aqueous amylose in conformation of a random flexible coil. By rapidly drying the aqueous amylose, the random flexible coil is ‘frozen’ giving rise to glass. The heating and drying are usually performed at the same step during the coating...
process. It is the microstructure of the glass that is thought to be responsible for resistance to the action of pancreatic α-amylase. The microstructure becomes more permeable when the glass is heated through the glass transition and becomes rubbery. For pure amylose, the glass transition temperature is very high and amylose thermally degrades prior to the temperature being reached. The glass transition can be reduced by adding ethylcellulose (Milojevic et al 1996a).

Ethylcellulose is an ethyl ether of cellulose containing 44-51% of ethoxyl groups (OC\(_2\)H\(_5\)). It is insoluble in water, but soluble in chloroform and alcohol. It is possible to form water soluble grades with a lower degree of substitution. Two grades were used: N-50 and N-100, both obtained from Hercules Incorporated, Wilmington, Delaware, U.S. Viscosity, determined using 5% ethylcellulose in 80 parts toluene and 20 parts ethanol by weight, was 40-52 and 80-105 cps for the N-50 and N-100 grades respectively. Ethylcellulose is used to coat one or more active ingredients of a tablet to prevent them from reacting with one another. It is also used to prevent discolouration of easily oxidisable substances such as ascorbic acid or to mask the bitter taste of drugs (Hercules technical brochure).

Sebacid Acid Dibutyl Ester (DBS) (C\(_{18}\)H\(_{34}\)O\(_4\)) was from Sigma-Aldrich Company Ltd (Poole, Dorset, UK, code S-2875). It is used in oral pharmaceuticals as a plasticizer for film coatings on tablets, beads and granules at concentrations of 10-30% by weight of polymer. It is also used as a synthetic flavour and flavour adjuvant in food products e.g. up to 5 ppm is used in ice cream and non alcoholic beverages. It is a clear, odourless, oily liquid with a bland to slight butyl odour. It is soluble in ethanol, propan-2-ol and mineral oil and practically insoluble in water (Wheatley 1994).

Fructooligosaccharide (Raftilose® P95, Raftiline® ST and Raftiline® HP) by Orafti Active Food Ingredients (Tienen, Belgium) was a gift from Dr G. Macfarlane, Dunn Institute of Clinical Nutrition, Cambridge. It is a natural substance belonging to a group of carbohydrates occurring in 36,000 different plants and it is composed primarily of fructose molecules. It is a dietary fibre most commonly found in onion, leek, garlic, banana,
artichoke and chicory root (Orafti technical brochure) which selectively activates the growth of *Bifidobacteria* and is safe for human consumption (Yazawa 1978, Okada et al 1984). Raftilose P95, Raftiline ST and Raftiline HP are a mixture of oligosaccharides which are composed of fructose units linked together by β(2-1) linkages. Part of these molecules are terminated by a glucose unit. The total number of fructose or glucose units (degree of polymerisation, or DP) of fructooligosaccharide ranges mainly between 2-8 for Raftilose P95, 2-60 for Raftiline ST and Raftiline HP. They are white fine powders with 800, 120 and 25 g / l solubility for Raftilose P95, Raftiline ST and Raftiline HP respectively (Orafti, Technical brochure).

2.1.2 Solvents

*Methanol* (CH$_3$OH), *Dichloromethane* (CH$_2$Cl$_2$), and *Ethanol* (CH$_3$CH$_2$OH) were of AnalaR grade and obtained from BDH Ltd, Poole, U.K.

2.1.3 Water

Except for cleaning purposes where tap water was used, all water used was distilled and provided by the School of Pharmacy’s, University of London main supply.

2.1.4 Media and nutrients

Nutrient Broth No. 2 (code CM67), nutrient agar (code CM3), MacConkey agar (code CM7), Wilkins-Chalgren (WC) agar (code CM643) and de Man, Rogosa, Sharpe (MRS) agar (code CM359) were obtained from Unipath Ltd (Basingstoke, Hants). When reconstituted their composition per litre was as follows:
### Chapter 2 Materials and Methods

#### Nutrient Broth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab Lemco powder</td>
<td>10 g</td>
<td></td>
</tr>
<tr>
<td>Peptone</td>
<td>10 g</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
<td></td>
</tr>
</tbody>
</table>

25 g of dry nutrient broth powder were dissolved in 1 litre of distilled water to give the above.

#### Nutrient Agar:

As nutrient broth, but containing in addition 15 g per litre Oxoid No3 agar.

#### MacConkey Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>20 g</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>10 g</td>
<td></td>
</tr>
<tr>
<td>Bile salts</td>
<td>5 g</td>
<td></td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5 g</td>
<td></td>
</tr>
<tr>
<td>Neutral Red</td>
<td>0.075 g</td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>12 g</td>
<td></td>
</tr>
</tbody>
</table>

50 g of dry MacConkey agar powder were dissolved in 1 litre of distilled water to give the above.
Chapter 2 Materials and Methods

<table>
<thead>
<tr>
<th>Wilkins-Chalgren Broth</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10 g</td>
<td>Tryptone</td>
</tr>
<tr>
<td>10 g</td>
<td>Gelatin peptone</td>
</tr>
<tr>
<td>5 g</td>
<td>Yeast extract</td>
</tr>
<tr>
<td>1 g</td>
<td>Glucose</td>
</tr>
<tr>
<td>5 g</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>1 g</td>
<td>L-Arginine</td>
</tr>
<tr>
<td>1 g</td>
<td>Sodium pyruvate</td>
</tr>
<tr>
<td>0.0005 g</td>
<td>Menadione</td>
</tr>
<tr>
<td>0.005 g</td>
<td>Haemin</td>
</tr>
</tbody>
</table>

33 g of dry Wilkins-Chalgren agar powder were dissolved in 1 litre of distilled water to give the above.

Wilkins-Chalgren Agar: as Wilkins-Chalgren broth, but containing in addition 15 g per litre Agar No. 1.

Fructooligosaccharide, as above, in 2% "/w quantities together with crude porcine mucin (M-2378) from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) also in 2% "/w quantities were added to Wilkins-Chalgren broth and agar to enhance bifidobacterial growth as suggested by Dr. G. Macfarlane (personal communication).
MRS Broth

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10 g</td>
<td>Peptone</td>
</tr>
<tr>
<td>8 g</td>
<td>'Lab-Lemco’ powder</td>
</tr>
<tr>
<td>4.0 g</td>
<td>Yeast extract</td>
</tr>
<tr>
<td>20 g</td>
<td>Glucose</td>
</tr>
<tr>
<td>1 ml</td>
<td>'Tween’ 80</td>
</tr>
<tr>
<td>2.0 g</td>
<td>K$_2$HPO$_4$</td>
</tr>
<tr>
<td>5.0 g</td>
<td>Sodium acetate 3H$_2$O</td>
</tr>
<tr>
<td>2.0 g</td>
<td>Tri-ammonium citrate</td>
</tr>
<tr>
<td>0.2 g</td>
<td>Magnesium sulphate 7H$_2$O</td>
</tr>
<tr>
<td>0.05 g</td>
<td>Manganese sulphate 4H$_2$O</td>
</tr>
</tbody>
</table>

52 g of dry MRS broth powder were dissolved in 1 litre of distilled water to give the above.

MRS agar as MRS broth, but containing in addition 15 g per litre Agar No1, which was obtained from Lab M (Bury, UK).

Preparation of Davis and Mingioli’s Salts Solution

To prepare Davis and Mingioli’s salts solution (DM base) the following were diluted in 2 l of distilled water in the order given (Davis & Mingioli, 1950):

<table>
<thead>
<tr>
<th>DM base</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.00 g</td>
</tr>
<tr>
<td>6.00 g</td>
</tr>
<tr>
<td>0.94 g</td>
</tr>
<tr>
<td>0.20 g</td>
</tr>
<tr>
<td>2.00 g</td>
</tr>
</tbody>
</table>
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Preparation of half strength peptone water:
Seven and a half grams of powder to prepare peptone water (code CM9 from Unipath Ltd, Basingstoke, Hants) were dissolved in 1 l of distilled water to create half strength peptone water containing 5 g peptone and 2.5 g NaCl per litre.

2.1.5 Bacterial strains

*Escherichia coli* was strain J5-3 *pro met* (Bachmann 1972) and the *Bacillus subtilis* strain was a laboratory isolate, both from the culture collection of Dr. R. J. Pinney, The School of Pharmacy University of London. *Staphylococcus saprophyticus* (MVK95S2) was from the collection of Dr M. V. Kelemen, The School of Pharmacy, University of London.

*A Bacillus subtilis* spore suspension (2.6 x 10^7 spores per 0.1 ml) was supplied by Amsco Finn-Aqua, Steris Scientific (Camberley, Surrey, UK).

*Lactobacillus acidophilus* was strain 1237 and *Bifidobacterium longum* was strain NCFB2716 kindly provided by Dr. G. Macfarlane, Dunn Institute of Clinical Nutrition, Cambridge. A freeze dried *Lactobacillus acidophilus* preparation containing 7.56 x 10^{10} colony forming units (cfu) per mg was prepared and provided by Dr. A. McBain again from the Dunn Institute of Clinical Nutrition, Cambridge.

The above organisms provided a selection of aerobic Gram positive bacteria and aerobic Gram negative bacteria as well as strict anaerobic and facultative anaerobic Gram positive organisms (Table 2.1):
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Gram staining</th>
<th>Oxygen Requirement</th>
<th>Size (µm)</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td>Positive</td>
<td>Aerobic</td>
<td>0.5 - 2.5 x 1.2 - 10.0</td>
<td>Straight rods, in pairs or chains</td>
</tr>
<tr>
<td>S. saprophyticus</td>
<td>Positive</td>
<td>Aerobic</td>
<td>0.5 - 1.5 diameter</td>
<td>Spherical irregular clusters, single or in pairs</td>
</tr>
<tr>
<td>E. coli</td>
<td>Negative</td>
<td>Aerobic</td>
<td>1.1 - 1.5 x 2.0 - 6.0</td>
<td>Straight rods, single or in pairs</td>
</tr>
<tr>
<td>B. longum</td>
<td>Positive</td>
<td>Strict anaerobic</td>
<td>0.5 - 1.3 x 1.5 - 8</td>
<td>Very irregular rods with branching, in V arrangement</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>Positive</td>
<td>Facultatively anaerobic</td>
<td>0.5 - 1.2 x 1.0 - 10.0</td>
<td>Straight regular rods</td>
</tr>
</tbody>
</table>

Table 2.1. Differential characteristics of the vegetative organisms studied (Holt et al, 1994).
2.2 Apparatus

Throughout the course of the experimental work the following apparatus was used. The list provided contains information of the equipment suppliers.

Mixing: Kenwood Chef mixer, from Kenwood UK.

Extruding:  

a. Ram extruder manufactured at the School of Pharmacy, University of London, operated via a Lloyds MX50 Materials Testing Machine, from Lloyds Instruments, Materials Testing Division, Southampton, UK.

Sheronising: Caleva 120 spheroniser, from G.B. Caleva Ltd, Dorset, UK.

Incubating: All incubators were without air circulation from Baird & Tatlock, Chadwell Heath, UK.

Drying of agar plates: Water jacket incubator from Hearson Laboratory Equipment, London, UK.

Oven Drying: Hot box oven size 1 with air circulation, from Gallenkamp, Loughborough, UK.


Sieve Shaking: Sieves and sieve shaker were from Endecotts Ltd, London, UK.

Tableting: Instron Testing Machine TT-CM, from Instron, High Wycombe, UK.
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Diametral Compression: Diametral compression machine model CT-40 from Enfineering Systems, Nottingham, UK.

Friability Testing: Roche Friabilator, manufactured at The School of Pharmacy, University of London.

Capsule filling: Hofliger Karg device, device manufactured at The School of Pharmacy, University of London.

Capsule rotation: Heidolph, from Lab-Plant Ltd, Huddersfield, U.K.

Capsule drying: 500 Watt hairdryer by Elton Sales, Hemel Hempstead, Herts, U.K.

Disintegration testing: Apparatus conforming to the British Pharmacopoeia 1998, Appendix XIIB, manufactured by Copley & Sons, Nottingham, UK.

Stirring liquids: Magnetic stirrer from Rodwell, Brentwood, UK and Vortex mixer from Hook Ltd, UK.

Dispensing: Automatic dispenser from Jencons Scientific, Leighton Buzzard, UK.


Centrifuging: Centrifuge models HS-18 and Centaur 2, both from M.S.E. Scientific Instruments, Crawley, UK.
2.3 Methods

2.3.1 Pellet Production

Where possible, equipment was sterilised by autoclaving before use. If this were not possible, it was swabbed with 70% ethanol and allowed to air dry.

All experiments were performed in triplicate to ensure their reproducibility.

2.3.1.1 Formulation

The pellet formulation used was derived by Harrison (1982) and was a mix of microcrystalline cellulose (Avicel PH101), Lactose BP and distilled water in the ratio of 5:5:6.

2.3.1.2 Mixing

A wet powder mass of the above formulation was prepared in 100 g batches, by mixing the powder excipients in a planetary type mixer (Kenwood Chef) for 5 min at the lowest setting of 100 rpm. A bacterial suspension in sterile distilled water (prepared as described in section 2.3.5) was then added and the mixing continued for a further 10 minutes. The mixing process of the wet mass was stopped at 2 minute intervals to scrape the sides of the bowl and blade with a sterile spatula so that an even distribution of liquid could be achieved.

Although Raines (1990) showed that masses containing Avicel PH101 should be sealed and stored for at least 4 hours to ensure complete equilibrium of the mixture this was not performed for the wet masses containing viable bacteria.

2.3.1.3 Extrusion

Two ram extruders were used: either a ram extruder manufactured at the School of Pharmacy operated via the Lloyds MX50 Materials Testing Machine or a Rheometric Scientific Acer Series 2000 Capillary Rheometer.
Extrusion using the Lloyds MX50

All formulation mixes were extruded using the Lloyds MX50 (Fig. 2.1 and Fig. 2.2) at a constant ram speed of 200 mm min\(^{-1}\) using a single hole die of 4 mm length and 1 mm diameter (i.e. a length to radius ratio of 8). The \(E.\ coli\) formulation was also extruded at ram speeds of 100 mm min\(^{-1}\) and 50 mm min\(^{-1}\) using the same die. The effect of die dimensions on the survival of \(E.\ coli\) was determined by extruding at a constant ram speed of 200 mm min\(^{-1}\) through single hole dies of 1 mm diameter and either 2 mm or 6 mm length (length to radius ratios of 4 and 12 respectively).

This ram extruder was manufactured in The School of Pharmacy, University of London, according to the design of Ovenston and Benbow (1968). A Lloyds MX50 Materials Testing Machine was used as a mechanical press to operate the ram extruder. The extruder consisted of a 20.5 cm long hardened steel barrel with an internal diameter of 2.5 cm and a wall thickness of 0.95 cm. The piston was 27 cm long and 2.5 cm in diameter. A PTFEE ring was placed 6 mm from the end of the piston to provide a low friction seal during the extrusion process. A rubber "O" - ring was placed in the groove on the bottom of the barrel which was then aligned with the groove in the die. The position of the holes were matched and the allen screws were tightened using the allen key. The wet mass was then added to the barrel and the large voids were removed by pushing a perspex plunger of a smaller diameter than the barrel into the barrel. This process was repeated until the wet powder mass level was 10 mm below the top of the barrel. The piston was then inserted into the barrel and the whole assembly was placed on a C - piece where collection of the extrudate took place, below the load cell. A single stainless steel barrel and a single set of stainless steel dies were used for all experiments to ensure reproducibility of results. The required ram speed (50, 100 or 200 mm min\(^{-1}\)) maximum force and displacement values were entered into the computer. The crosshead was lowered so that the piston head was in place. The extrusion force, measured by the load cell was plotted against the displacement to produce a force / displacement profile (Fig.2.3).
Fig. 2.1 Diagram of ram extruder operated via the Lloyds MX50 Materials Testing Machine.
Fig. 2.2 Lloyds MX50 Materials Testing Machine with ram extruder manufactured at The School of Pharmacy, University of London.
The formulation was packed in the barrel and forced through the die by the piston, operated via the mechanical press.
Fig. 2.3 Typical force/displacement profile. The extrusion force is measured by the load cell is plotted against the ram’s displacement.
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From the graph in Fig. 2.3, three distinct stages existed. The first stage was the compression stage where the wet mass was consolidated until all voids had been removed and a large plug was formed. The apparent density of the plug was found to be approximately equivalent to the apparent particle density of the wet powder mass (Harrison 1982). Next, the steady state stage occurred where the extrudate begun to be produced. The force required to produce a continuous flow of extrudate, was constant at this stage and therefore, resulted in a constant, uniform extrudate being produced. The final stage was the forced flow stage. At this stage an increase in force was required for the continuation of extrusion. The flow patterns within the barrel were altered and the extrudate produced was no longer uniform in nature. This point signified the end of the extrusion process. Force/displacement profiles have been found to be dependent upon the length to radius of the die used, the materials being extruded and the speed of extrusion (Harrison 1982).

Extrusion using the Rheometric Scientific Acer Series 2000 Capillary Rheometer

Capillary extrusion rheometers work by extruding material at a known and controlled temperature through an orifice of known geometry. The volume of material extruded at a given time and the pressure required to do this are measured throughout the process. The Rheometric Acer Series 2000 Capillary Rheometer was used as a ram extruder in experiments to determine whether the distribution of water in the extrudate and the extrusion force applied affected the survival of \textit{E. coli}. A batch of the \textit{E. coli} formulation was extruded at a low extrusion speed to maximise water movement (50 mm min$^{-1}$) through the Acer extruder. While the material is forced through the die of 1 mm diameter, 10 mm length and 30$^\circ$ angle the pressure required for the extrusion to take place is monitored continuously by a pressure sensor located above the die. The barrel orientation allows the extrudate to be sampled (Fig. 2.4): due to the absence of a C-piece, the operator can change the collection beaker at pre set time intervals so that samples can be collected during the extrusion process. Eight extrudate samples were taken at 15 sec intervals. The water content present in each sample was measured and the extrusion force read from the pressure / displacement profile, which this equipment stores on a computer hard disc.
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Fig. 2.4 Rheometric Scientific Acer Series 2000 Capillary Rheometer.

Mechanical Press

Piston

Barrel

Collection Point
2.3.1.4 Spheronisation
All extrudates were spheronised at a constant speed of 1883 rpm for 5 min using a laboratory scale spheroniser (Caleva 120) with a radial geometry plate of 12 cm diameter. The pellets were then removed via the collection shoot.

2.3.1.5 Air drying
The pellets were dried in an incubator, at 25° C for 48 h, after which their weight was constant.

The water content of extrudate samples collected from the Acer extrusion was determined by drying them in an oven at 60° C overnight, after which their weight was constant. The samples were weighed before and after drying. The measured weight difference represents the water content of each sample tested.

2.3.1.6 Freeze drying
One ml samples of overnight cultures or “washed” bacterial suspensions, or one gram of mix, extrudate or pellet samples containing the anaerobic organisms were placed into 1 fl. oz. screw cap glass bottles and frozen for 4 hours in a -4° C freezer, from Payne Scientific, UK. Due to the small volume of the samples, “shell freezing” was not necessary to speed up the freeze drying process. The samples were then freeze dried in a Micro Modulyo freeze drier designed to operate via an Edwards RV3 one phase pump. The freeze drier used had a high capacity air cooled refrigeration compressor and a stainless steel condenser. The refrigeration system operated at temperatures down to -55° C and the condenser chamber was thermally insulated. An azide-proof vacuum pipeline connected the chamber to a tube on the front panel, to which the pump was connected. The Edwards RV3 one phase pump had a maximum water vapour pumping rate of 0.06 kg / hr.

2.3.2 Tablet production
To reduce particle size, freeze dried agglomerates of L. acidophilus were milled in a 220 ml
capacity and 100 mm external diameter glass mortar for 1 min using a 135 mm long glass pestle. This was then sieved through a nested set of sieves which conformed to B.S.410 and arranged in a \( \sqrt{2} \) progression, for 20 min, using a mechanical sieve shaker. This particle size reduction was necessary to ensure homogeneous mixing with the smaller particle size excipients of the tablet formulation.

Two g of freeze dried *L. acidophilus* (particle size 90 - 125 \( \mu \)m), were hand mixed with 2 g of Ac-Di-Sol, as disintegrant, for 30 sec in a porcelain glazed 750 ml capacity and 163 mm external diameter mortar using a 215 mm length porcelain glazed pestle. Six g of the direct compression excipient, Avicel PH-101 were then added and the mixing continued for another 30 sec. Finally 90 g of Avicel PH-101 were added and mixed for 30 sec to give a formulation mix of 2 % *L. acidophilus* and 2 % Ac-Di-Sol in Avicel PH-101.

A similar tablet mix was also prepared with another direct compression excipient, FlowLac. Half a gram of magnesium stearate was mixed with 0.5 g of FlowLac using the porcelain glazed pestle and mortar as above for 30 sec. In 30 sec intervals, a further 1 g, 2 g, 4 g, 8 g, 9 g of Flow Lac were added.

Due to the limited supply of freeze dried *L. acidophilus*, 40 ml of a *L. acidophilus* suspension (prepared as described in section 2.3.5) was hand mixed, with a sterile metal spatula in a sterile 250 ml capacity beaker for 15 sec into the FlowLac and magnesium stearate powder mix. Both spatula and beaker had been heat sterilised at 150°C for 1 hour. The addition of 2% w/w magnesium stearate was necessary to provide the adequate lubrication for the tablets to be ejected out of the die after compaction. This tablet mix was then freeze dried.

One hundred mg of the Avicel/Ac-Di-Sol tablet mix and 200 mg of the FlowLac/magnesium stearate tablet mix were poured into an 8 mm diameter die. They were compressed between flat faced punches at a rate of 1 mm min\(^{-1}\) to the required pressure (50 - 300 MPa) by an Instron Testing Machine Model TT-CM and maintained at this value for 30 sec.
2.3.2.1 Breaking load

The breaking load of the tablets produced at pressures of 50 - 300 MPa as described above was measured. The tablets were loaded in a CT-40 tablet diametral compression machine. The span between the centre of the support was 45 mm. Loads were applied at crossheads of 1 mm min⁻¹ and the breaking load was read from the display.

2.3.2.2 Friability testing

Five tablets produced by compaction at 50 MPa were weighed and loaded in a Roche Friabilator. The device was operated for 20 min. The tablets were collected and weighed at 5 min intervals and at the end of the test for the weight loss to be determined.

2.3.3 Capsule production

2.3.3.1 Capsule filling

Twenty five grams of a FlowLac/magnesium stearate formulation mix containing freeze dried *L. acidophilus* was prepared as described in section 2.3.2. This was used to fill size 0 hydroxypropyl methylcellulose (HPMC) capsules (Qualicaps from Shionogi, Madrid, Spain) in 500 mg quantities. A Hofliger and Karg device for determining the volume occupied by a formulation under a known pressure was used to fill the capsules (Fig. 2.5). It comprised of a 6 mm diameter die, where the powder was placed, and a 6 mm diameter piston. Because of the small die size, compaction of 500 mg of formulation mix in one step was not possible. Therefore, powder compaction was performed in two stages: 250 mg formulation mix were compacted, ejected into the capsule body and then a further 250 mg of formulation mix were compacted and added into the capsule. The powder was compressed until the pressure reached 2 N. Finally, the capsule cap was firmly placed on top. Capsules were weighed before and after filling, to check for any powder mix losses during filling.
Fig. 2.5 **Hofliger Karg device** used for filling capsules. The formulation powder was compressed until the pressure reached 2 N. The powder plug was then ejected into the capsule body and the capsule cap firmly placed on top.
2.3.3.2 Capsule coating

Preparation of coating solutions

Two coating solutions were prepared: an ethylcellulose solution and an ethylcellulose /amylose solution:

To prepare the ethylcellulose solution, a solvent mix of 50:50 \(^{\text{w/w}}\) mixture of methanol and dichloromethane was initially made, by mixing 12.5 g of each solvent with a magnetic stirrer for 10 minutes. Then, 2.5 g of ethylcellulose N-50 and 0.75 g of PEG 4000 were added and the mixing continued for a further one hour, after which a viscous solution was formed.

To prepare the ethylcellulose /amylose solution, a 6 \(^{\text{w/w}}\) solution of ethylcellulose in absolute alcohol was initially made, by mixing 2 g ethylcellulose N-100 and 33.333 g absolute alcohol with a magnetic stirrer for 3 hours. Then, 0.7 g of DBS were added and the mixing continued for a further 10 minutes. Finally, 13.33 g of amylose 5 \(^{\text{w/w}}\) solution were added and the mixing continued for a further one hour, after which a viscous solution was formed.

Coating

A filled capsule was rotated at 330 rpm by the action of a Heidolph controlled speed stirrer. Half the capsule was left uncovered by the rubber adaptor (fig. 2.6) and was immersed in the coating solution. A tissue was used to wipe off excess. Where stated, heat (50\(^{\circ}\) C) generated by means of a hairdryer was used to dry the coat. After the coat had dried, the capsule was removed, weighed and the weight gain was recorded. The capsule was then once again placed in the rotation device, this time turned upside down so that the uncoated part of the capsule was exposed, and the procedure repeated as above. Subsequent applications of coating solution were necessary to provide acceptable coat thickness.
Fig. 2.6 **Capsule coating.** Each capsule was placed in a rubber capsule adaptor which was rotated by the action of a controlled speed stirrer. The exposed part of the capsule was immersed in the coating suspension and where stated, dried by the action of a hairdryer. The coating suspension was kept under constant stirring to prevent precipitation.
2.3.3.3 Disintegration testing for coated capsules

The British Pharmacopoeia 1998 disintegration test for enteric-coated tablets (British Pharmacopoeia 1998b) was applied to the ethylcellulose/amylose coated capsules. Six capsules of each coat thickness were tested. They were subsequently immersed into three different types of media: simulated gastric and intestinal media and a third medium containing amylase, which is an enzyme secreted from the normal intestinal flora present in the colon. Simulated gastric medium was made by making a 0.32 % pepsin (from BDH Laboratory Supplies, Poole, UK) solution in 0.1 N hydrochloric acid. Simulated intestinal medium was made by making a 1 % pancreatin (from BDH Laboratory Supplies, Poole, UK) solution in phosphate buffer of pH 6.8. The final medium was 0.2 % amylase (from Sigma Chemical Co) in phosphate buffer pH 6.8. After immersion, a mechanical device raised and lowered the disintegration apparatus, through a distance of 50 to 60 mm at a constant frequency of between 28 and 32 cycles per minute. The temperature was maintained at 37°C by a water bath, which was build in the disintegration apparatus.

The simulated gastric and intestinal media were applied for 3 and 4 hours respectively, whereas the amylase medium was applied for 8 hours. The coated capsules passed the disintegration test if all six remained intact after the application of the gastric and the intestinal media and disintegrated in the amylase medium.

2.3.4 Preparation of media

Preparation of broths

For the reconstitution of each broth the weight of powder specified by the manufacturer (Tables, Part 2.1 Materials) was carefully weighed into a 2 l capacity conical flask (B.S.2734). The powder was dissolved by the action of a magnetic stirrer. After reconstitution broths for the growth of aerobic organisms were dispensed using an automatic dispenser, which was calibrated each time before use, in 4.5 ml volumes into 1 fl. oz. capacity bottles with screw caps. For the growth of anaerobic organisms 500 ml capacity glass bottles with screw caps were filled to the brim with MRS or WC broth. Using this
method, it was not necessary to degas the bottles to produce a sufficient bacterial yield. After dispensing, all broths were sterilised by autoclaving at 10 psi (115°C) for 15 min prior to use.

*Preparation of agar plates*

For the reconstitution of each agar the amount specified in Tables, Part 2.1 Materials section was carefully weighed into 4 fl. oz. screw cap glass bottles and 100 ml of distilled water measured with a graduated cylinder was added. The bottles were then shaken to achieve a homogenous suspension.

After reconstitution the bottles were sterilised by autoclaving at 10 psi (115°C) for 15 min prior to use. After autoclaving they were placed in an incubator at 55°C to cool. Approximately 20 ml volumes of the hot agar were poured into triple vent sterile petri dishes of 90 mm diameter (code G33185, from Greiner Labortechnik, Stonehouse, UK). When anaerobic organisms were incubated, the vented petri dishes allowed anaerobic conditions to be generated between the agar surface and the petri dish lid. After 30 min the agar had set and the plates were dried inverted with lid removed in the water jacket incubator for 45 min at 55°C.

*pH adjustment*

The pH of the MRS broth and agar was adjusted to the value of 6 for optimum *Lactobacilli* growth as indicated by Dr. G. Macfarlane (private communication). A PTI-15 pH meter, fitted with a Gelplas epoxy polymer-bodied gel-filled electrode supplied by Fisher Scientific, Loughborough, UK, was used. Standardisation was by means of two buffer solutions of known pH (pH 7.00 ± 0.01 at 25°C, code B-4770 and pH 4.00 ± 0.01 at 25°C, code B-5020) obtained from Sigma Chemical Co. Hydrochloric acid (HCl) at a concentration of 0.02 M was added to the MRS broth and agar to adjust their pH to 6. It was prepared by diluting hydrochloric acid ‘AnalaR’ (code 10125LB, from BDH Merck Ltd, Lutterworth, UK). Drops of the 0.02 M HCl solution were dispensed via a Pasteur pipette (145 mm length, dispensing 30 to 35 drops per ml, operated via a rubber teat and manufactured by John Poulten Ltd,
Chapter 2 Materials and Methods

Barking, UK) and were mixed by the action of the magnetic stirrer (as above) until the pH measured was 6.

Preparation of diluents

Distilled water, half strength peptone water and Davis & Mingioli’s basal salts solution were prepared in 1 l quantities as described in the Materials section 2.1.3. They were dispensed in 4.5 and 9.9 ml volumes using the automatic dispenser (as above) into 1 fl. oz. capacity glass bottles with screw caps. The bottles were then sterilised by autoclaving at 10 psi (115°C) for 15 min prior to use.

2.3.5 Preparation of bacterial suspensions

All organisms tested were stored at -135°C in liquid nitrogen. Prior to use, they were thawed and a loop-full was aseptically plated on the relevant agar for each organism (Table 2.2). After 2 days incubation at 37°C under aerobic or anaerobic conditions according to the organism (Table 2.3), a single colony was aseptically transferred into the relevant broth (Table 2.2). For the aerobic organisms the colony was suspended in the broth using a Vortex mixer for 15 sec to ensure optimal growth, whereas this was not necessary for the anaerobic organisms. Three overnight incubated 4.5 ml broth cultures were sufficient to prepare an inoculum for a batch of pellets at approximately $10^8$ colony forming units per gram. However, one 500 ml broth culture incubated overnight for L. acidophilus was necessary to produce the same count of cfu per gram of formulation mix. Whereas, the 500 ml broth culture of Bifidobacterium longum had to be incubated for 48 hours to produce similar counts in the pellets.
Table 2.2: Broth type and volume used for the growth of the microorganisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Broth / Agar</th>
<th>Broth volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>nutrient</td>
<td>13.5</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>nutrient</td>
<td>13.5</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>nutrient</td>
<td>13.5</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>de Man Rogosa Sharpe</td>
<td>500</td>
</tr>
<tr>
<td><em>B. longum</em></td>
<td>Wilkins - Chalgren</td>
<td>500</td>
</tr>
</tbody>
</table>

In addition, the anaerobic bacteria were incubated under anaerobic conditions produced in an anaerobic jar of 1.5 l capacity by a gas generating kit of sodium borohydrate, tartaric acid and sodium bicarbonate (code BR38, supplied from Unipath Ltd, Basingstoke, Hants, UK). Ten ml of distilled water measured in a 10 ml capacity graduated cylinder (B.S.604) were added to the gas generating sachet which then generated approximately 1800 ml hydrogen and 350 ml carbon dioxide. Gas generation was completed within 30 minutes. The volume of hydrogen produced was adequate to ensure virtually complete anaerobiosis in the presence of a palladium catalyst (code BR42, supplied from Unipath Ltd, Basingstoke, Hants, UK) in the jar used. The catalyst acted as an efficient heat sink so that the maximum temperature in the jar was lower than the flash point of hydrogen gas. Explosions in anaerobic systems are rare and can occur when hydrogen and oxygen are present in critical proportions. Such explosions are triggered by extremely hot catalyst temperature. The catalyst contained 4 g of palladium, following the recommendation of the UK Department of Health and Social Security that there should be not less than 1 g of catalyst for each litre volume of anaerobic jar (Unipath technical brochure).

The reconstituted gas generating sachet and the catalyst were placed together with one 500 ml broth bottle in the jar. The bottle screw cap was screwed on loosely, so that the hydrogen and carbon dioxide gasses could enter in any head space the bottles still had. The jar was
then sealed securely by a layer of DC4 silicone compound (Dow Corning, Wiesbaden, USA) between the jar and it’s lid and placed in the incubator for the relevant time (Table 2.3).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Environment</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>aerobic</td>
<td>$37^0\text{C}$</td>
<td>16 hours</td>
</tr>
<tr>
<td>S. saprophyticus</td>
<td>aerobic</td>
<td>$37^0\text{C}$</td>
<td>16 hours</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>aerobic</td>
<td>$37^0\text{C}$</td>
<td>16 hours</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>anaerobic</td>
<td>$37^0\text{C}$</td>
<td>16 hours</td>
</tr>
<tr>
<td>B. longum</td>
<td>anaerobic</td>
<td>$37^0\text{C}$</td>
<td>48 hours</td>
</tr>
</tbody>
</table>

Table 2.3: Incubation conditions used for the growth of the microorganisms.

After incubation, the cultures were transferred aseptically into sterile plastic containers with screw caps tightly fitted on, and centrifuged at 4000 rpm for 20 min. For the smaller 13.5 ml volumes of the aerobic organisms sterile universal containers (code 30593, from L.I.P. Equipment & Services, Shipley, UK) were used in the Centaur 2 centrifuge. For the the larger 500 ml volumes of L. acidophilus and B. longum cultures, 250 ml were poured into two 290 ml capacity autoclavable plastic bottles (code CFS-128-F, from Fisher Scientific, Loughborough, UK) with screw cap, which were previously sterilised by autoclaving at $115^0\text{C}$ for 15 min. Since sterilisation of empty sealed containers by autoclaving is not feasible, a drop of water was placed in each bottle before autoclaving to generate the necessary pressure for sterilisation. The bacterial cultures were spun in a HS-18 centrifuge at 4000 rpm for 20 min. Equal volumes of distilled water in similar containers were positioned opposite the cultures to balance their weight during the centrifuge operation. After centrifugation, the supernatant was discarded and the cultures were resuspended in equal volumes of sterile distilled water and centrifuged again at 4000 rpm for 20 min. The supernatant was again discarded and the pellets finally suspended in 20 ml sterile distilled water before inoculation of the pellet formulation mix to give approximately $10^8$ cfu per gram.
2.3.6 Viable counting

Viable counts were performed on samples of the “washed” bacterial suspensions and on samples of the formulations after mixing, extrusion, spheronisation and drying. Samples of the anaerobic broth cultures were also counted before and after washing. The viable counts per gram of the tablets produced were also determined.

The liquid samples were 1 ml and were measured using an automatic pipette (by Gilson, France model P1000 and of range 0.2 - 1 ml), whereas the solid samples were 1 gram and were measured by weighing. However, when the viable count of tablets and capsules was examined, whole tablets, after weighing, and a single capsule torn with a sterile razor to release its contents, constituted a single sample. All samples were suspended using a vortex mixer (as before) for 30 sec into 10 ml of DM base in the case of aerobic organisms or in half strength peptone water when anaerobic organisms were counted.

The resultant suspension was serially diluted, in DM or half strength peptone water, using 1 in 10 and 1 in 100 dilutions: that is 0.5 ml of sample suspension in 4.5 ml diluent and 0.1 ml of sample suspension in 9.9 ml diluent respectively (using the Gilson pipette, as before). Viability was determined using the viable counting technique of Miles and Misra (1938): Five 20 μl volumes of each dilution were measured with an automatic pipette Gilson (France) model P200 of volume range 0.05 - 0.2 ml, placed on the relevant agar for each organism (Table 2.2) and incubated at 37°C to produce colonies. Overnight incubation was adequate for all organisms, whereas, 2 days incubation were necessary for B. longum to develop colonies. Both anaerobic L. acidophilus and B. longum plates were incubated under anaerobic conditions, generated as described before.

2.3.6 Statistical analysis

All statistical calculations were done using the computer package “Excel” version `97 (by Microsoft Corporation, USA).
2.3.6.1 Significance tests

Significance tests were employed to test whether the difference between results was significant, or was due to random (experimental) variations. The following statistical calculations test the truth of a null hypothesis. The term null hypothesis implies that there is no difference between the observed and the known values other than that which can be attributed to random variation.

Assuming that the null hypothesis was true, statistical theory was used to calculate the probability that the observed difference between a sample mean and the true value arose solely as a result of random errors. The lower the probability that the observed difference occurred by chance, the less likely it was that the null hypothesis was true. The null hypothesis was rejected if the probability \( P \) of such a difference occurring by chance was less than 1 in 20, i.e. 0.05 or 5%. In such a case the difference between two results was said to be significant at the 0.05 level \( P < 0.05 \).

By using the computer statistical package “Excel” the critical values of the \( t \)-test and ANOVA were calculated, \( t \) and \( F \) respectively. If those values exceeded the critical values, \( t_{\text{crit}} \) and \( F_{\text{crit}} \) respectively, then the null hypothesis was rejected.

**t-test**

Comparison of the means of two samples was performed using the \( t \)-test. This test determined whether two sample means were likely to have come from the same two underlying populations that had the same mean.

In the following experiments, when the difference between a pair of means had to be tested for significance, for example bacterial counts before and after a process, a paired \( t \)-test was applied. However, a paired test could not be applied if the sample means were not paired, for example if the bacterial counts came from different batches. Then a two or one tailed \( t \)-test was used.

If there was no indication prior to experimental measurements as to whether any significant difference between the experimental means would be positive or negative, the test had to
cover either possibility: such a test is called two-tailed (or two-sided) and was used in the following experiments to compare the effect of different drying methods on bacterial survival.

However, in experiments where bacterial survival was tested for significance, it was clear before the experiments began that the only result possible was whether the new bacterial viable counts were less than the old, and thus only a decrease in the number of bacteria was tested for significance, therefore, a one-tailed (one-sided) test was done.

The two sample variances, i.e. the squares of the standard variations, were unequal in the following experiments, so the $t$-tests performed were heteroscedastic, as opposed to a homoscedastic $t$-test which assumes that the variances of both data sets, and subsequently their means, are equal.

Analysis of variance (ANOVA)

ANOVA was performed when more than two sample means were to be compared. It can separate two different sources of variation: the random error in each measurement, which is always present, and the controlled or fixed-effect factor, which in the following experiments was defined as an extrusion variable.

2.3.6.2 Correlation analysis

To test whether two variables were related the product moment correlation coefficient, $r$ or otherwise known as correlation coefficient was calculated. The correlation coefficient can take values in the range $-1 \leq r \leq +1$. A value of -1 describes perfect negative correlation, i.e. all the experimental points lie on a straight line of negative slope (Fig. 2.8) and the two variables are negatively correlated. Similarly, when $r = +1$ the correlation is perfect positive, i.e. all the points are lying exactly on a straight line of positive slope (Fig. 2.7) and the two variables are positively correlated. When there is no correlation between the two variables the value of $r$ is zero (Fig. 2.9).

In practice values of 1 or zero are rarely experimentally obtained and the value of $r$ lies
between zero and one. Such values were obtained in the following experiments and it was necessary to use a significance test to see whether the correlation was in fact significant, bearing in mind the number of pairs of points used in the calculation. This was achieved by a paired $t$-test at the significance level (probability) of $P < 0.05$. The null hypothesis in this case was that there was no correlation between the two variables. If the calculated value of $t$ was greater than the tabulated value, the null hypothesis was rejected, i.e. the two variables were significantly correlated.
Fig. 2.7 Perfect positive correlation between variables x and y ($r = +1$).

Fig. 2.8 Perfect negative correlation between variables x and y ($r = -1$).

Fig. 2.9 Zero correlation between variables x and y ($r = 0$).
Chapter 3:

**Bacterial Survival During Extrusion - Spheronisation**
Chapter 3 Bacterial Survival during Extrusion - Spheronisation

3.1 Introduction

The feasibility of incorporating bacteria into pellets produced by extrusion-spheronisation was investigated using both model and probiotic organisms. Gram-negative aerobic (Escherichia coli), Gram-positive aerobic (Staphylococcus saprophyticus and Bacillus subtilis) and Gram-positive anaerobic (Lactobacillus acidophilus and Bifidobacterium longum) vegetative bacteria, together with spores of Bacillus subtilis, were introduced separately into a formulation, which was extruded, spheronised and dried to produce pellets, as described in Materials and Methods, section 2.3.1. Samples were taken at each stage of the pellet production process and checked for bacterial survival.

Unless otherwise indicated, bacterial survival was expressed as a percentage of the colony forming units (cfu) per gram of the initial inoculum. The number of cfu were counted after samples were diluted, plated on a suitable solid medium, and incubated, as described in Materials & Methods, section 2.3.5. The limit of detection of the counting method was $10^3$ cfu/g of sample, or where results are expressed as percentages it was 0.02% of the initial inoculum.

E. coli and S. saprophyticus grew on MacConkey agar producing typical pink colonies. B. subtilis produced flat creamy irregular colonies on nutrient agar, whereas B. longum gave flat white colonies on Wilkins-Chalgren agar. L. acidophilus produced cream colonies on MRS agar. No atypical, contaminating bacteria were observed during any of the counting processes.

Mean survival was determined from the average of three replicate experiments. For each experiment, three samples of formulation mix, extrudate, or pellets were taken and the viable counts determined as described in Material & Methods, section 2.3.5. Fig. 3.1 illustrates sampling of the first replicate, Batch A. Three samples of this batch were diluted and plated to give countable numbers of the inoculating organism. Three replica counts (drops 1, 2 and 3) were determined from samples 1, 2 and 3. Their average drop counts ($M_1$, $M_2$ and $M_3$) were used to calculate the mean viable count $M_A$ from batch A. Similar means ($M_B$ and $M_C$) were calculated for batches B and C respectively to give the grand mean of the experiment, M.
Fig. 3.1 Sampling of Batch A. Three samples of this batch were taken and plated to give countable numbers of the inoculating organism. Three replica counts (drops 1, 2 and 3) were determined from samples 1, 2 and 3. The average drop counts \( (M_1, M_2, \) and \( M_3 \)) were used to calculate the mean viable count \( M_A \) from batch A. Similar means \( (M_B \) and \( M_C) \) were calculated for batches B and C respectively to give the grand mean of the experiment, \( M \).
3.2.1 Survival of *Bacillus subtilis* spores

Bacterial spore coats act as a barrier to dehydration and provide mechanical strength thus rendering spores a highly resistant microbial form. Therefore, as a control, spores of *Bacillus subtilis* were introduced in a batch of extrusion mix and processed to produce pellets. As expected, it was found that *B. subtilis* spores survived the entire extrusion-spheronisation process. Even after air drying, the pellets were found to contain 100 per cent of the viable spores introduced into the mix (Fig. 3.2). Due to shortage of the spore suspension this experiment was only performed once, so it was not possible to calculate the standard deviations of the data obtained.

![Graph showing survival of *Bacillus subtilis* spores](image)

Fig. 3.2 Survival of *Bacillus subtilis* spores after mixing, extruding at 200 mm min\(^{-1}\) through a 4 mm radius and 1 mm diameter die, spheronising and air drying. Results are presented as logarithmic percentages of the viable counts present in the formulations before mixing.
3.2.2 Survival of vegetative cells of *Bacillus subtilis*

Compared to their spore form, vegetative cells of the Gram positive rod *Bacillus subtilis* were found to be sensitive to the extrusion-spheronisation process when their survival means were compared: An average of 41% of the initial inoculum survived extrusion, with 30% surviving after spheronisation and 13% still viable after air drying (Fig. 3.3). Compared to the initial inoculum, samples taken after the initial mixing of the “washed” *B. subtilis* suspension with the powders did not show any significant bacterial kill\(^1\).

![Graph showing survival of vegetative cells of Bacillus subtilis](image)

**Fig. 3.2** Survival of vegetative cells of *Bacillus subtilis* after mixing, extruding at 200 mm min\(^{-1}\) through a 4 mm radius and 1 mm diameter die, spheronising and air drying. Results are presented as logarithmic percentages of the viable counts present in the formulations before mixing.

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\(^{1}\) Only a reduction of viable count is tested for significance, therefore a one sampled, one tailed t-test \((P < 0.05)\) was used to compare the mean viable count of vegetative *B. subtilis* in formulation mix with the mean *B. subtilis* inoculum.
3.2.3 Survival of vegetative cells of *Staphylococcus saprophyticus*

The survival of *Staphylococcus saprophyticus* during the pellet production process was found to be similar to that of vegetative cells of *B. subtilis*: no significant loss in viability was produced by the mixing process\(^2\), with averages of 60 %, 57 % and 16 % showing loss of viability after extrusion, spheronisation and air drying respectively (Fig. 3.4). The similarity in the survival of *S. saprophyticus* and *B. subtilis* vegetative cells is likely to be due to the similar Gram-positive coat that surrounds both bacteria. The only difference between them is morphological: organisms of the genus *Staphylococcus* are coccus-shaped whereas these of the genus *Bacillus* are rod-shaped.

![Fig. 3.4 Survival of vegetative cells of *Staphylococcus saprophyticus* after mixing, extruding at 200 mm min\(^{-1}\) through a 4 mm radius and 1 mm diameter die, spheronising and air drying. Results are presented as logarithmic percentages of the viable counts present in the formulations before mixing.](image)

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\(^2\) As in section 3.2.3, a one tailed, one sampled \(t\)-test was performed \((P < 0.05)\).
3.2.4 Survival of vegetative cells of *Escherichia coli*

*Escherichia coli* is rod-shaped, like *B. subtilis*. However, unlike *B. subtilis* and *S. saprophyticus*, it does not have a Gram-positive cell wall. This Gram-negative organism was found to be much more sensitive to the extrusion-spheronisation process when compared to the Gram-positive *B. subtilis* and *S. saprophyticus*. Again, no significant reduction in viability was observed after mixing, but only 10% and 0.2% of the initial inoculum were viable after extrusion and spheronisation respectively, with no viable *E. coli* detected after air drying (limit of detection 0.02%), (Fig. 3.5).

![Graph showing survival of vegetative cells of *Escherichia coli* after mixing, extruding at 200 mm min⁻¹ through a 4 mm radius and 1 mm diameter die, spheronising and air drying. Results are presented as logarithmic percentages of the viable counts present in the formulations before mixing.]

Fig. 3.5 Survival of vegetative cells of *Escherichia coli* after mixing, extruding at 200 mm min⁻¹ through a 4 mm radius and 1 mm diameter die, spheronising and air drying. Results are presented as logarithmic percentages of the viable counts present in the formulations before mixing.

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As in sections 3.2.2 and 3.2.3, a one tailed, one sampled *t*-test was performed (*P* < 0.05).
3.3 Effect of extrusion parameters on the viability of *Escherichia coli*

The pellet production process, as described in *Material & Methods* section 2.2.1, is divided into four stages: mixing the ingredients of the formulation, extrusion of the formulation to produce spaghetti-like extrudate, spheronisation of the extrudate into spherical shaped pellets and finally air drying. It was shown in sections 3.2.1, 3.2.2, 3.2.3 and 3.2.4 that mixing had no significant effect on the viability of any of the model microorganisms. In an attempt to optimize bacterial survival after extrusion, the effects of varying the extrusion parameters (ram speed and die dimensions) were determined. *Escherichia coli* was used as the test organism, since it was found to be the most sensitive organism to extrusion (section 3.2.4), and therefore might be expected to give widest variations in survival level when the extrusion parameters were varied.
3.3.1 Ram speed

Extrusion of the *E. coli* formulation through a 4 mm long and 1 mm diameter die at ram speeds of 50, 100 or 200 mm min\(^{-1}\) gave survival levels of 23.5%, 16.3%, and 11.0% respectively (expressed as a percentage of the viable count after mixing), (Fig. 3.6). It might appear that survival is decreased by increasing ram speed. However, the differences in survival were not statistically significant\(^4\).

The fact that increasing the speed of ram extrusion has no effect on bacterial survival may be because the barrel wall shear stress is not much greater at 200 mm min\(^{-1}\) than at 50 mm min\(^{-1}\) (Fielden *et al.* 1987). The results are in agreement with those of Blair (1989) who showed that the survival of another Gram-negative rod, *Enterobacter cloacae*, was not altered when formulations similar to those used in this thesis were extruded at ram speeds of 50 or 400 mm min\(^{-1}\).

![Graph showing survival (%) vs. ram speed (mm/min)](image)

**Fig. 3.6 Effect of ram speed on the survival of *Escherichia coli.*** No significant effect on the viability of *E. coli* was demonstrated when mixes were extruded at ram speeds of 50, 100 and 200 mm min\(^{-1}\).

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\(^4\) More than two means were compared: mean *E. coli* survival after extrusion at 50, 100 and 200 mm min\(^{-1}\). Therefore, analysis of variance (ANOVA) was performed. The calculated *F* value was less than the critical value *F*\(_{crit}\), *P* < 0.05. The null hypothesis, that the sample means do not differ significantly, is thus accepted.
3.3.2 Die dimensions

Again, using the most sensitive of the model organisms, *E. coli*, it was found that extrusion of a mix through dies of length to radius ratios of 4, 8 or 12 gave 19.3 %, 11.0 %, and 17.7 % survival respectively in the extradates, expressed as a percentage of the viable count after mixing (Fig. 3.7). Statistical analysis of these data showed no significant differences in survival after extrusion through the different dies.

![Fig. 3.7 Effect of die length to radius ratio on the survival of *Escherichia coli*.](image)

No significant effect on the viability of *E. coli* demonstrated when mixes were extruded through dies of length to radius ratios of 4, 8 or 12.

3.3.3 Variation in viable count with water content of extrudate

The high standard deviations of the survival levels after extrusion at low ram speeds (Fig. 3.6) are due to a phenomenon described by Fielden *et al* (1992) as “water movement”: at lower extrusion speeds, the liquid content of a formulation is forced out of the die more quickly than the solid. Since the majority of bacteria are in aqueous suspension, it might be expected that early samples of extrudate will contain significantly higher numbers of

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5 As in section 3.3.1, analysis of variance (ANOVA) between the means was conducted (*P* < 0.05).
organisms than later samples.
To investigate this, a batch of formulation mix, inoculated with *E. coli* as before, was extruded at the lowest extrusion speed (50 mm min\(^{-1}\)) to maximise water movement. The extrudate was sampled every 15 seconds throughout the extrusion process, to give a total of eight samples. These were examined for water and *E. coli* content.
The water content of the formulation after mixing was 37.5%. Early extrudate samples had a higher water content than this, with a high of 43.0%. Later samples contained less water, with a low of 33.2% (Fig. 3.8).

![Graph showing variation in water content of *Escherichia coli* extrudate](image)

**Fig. 3.8 Variation in the water content of *Escherichia coli* extrudate** during extrusion at 50 mm min\(^{-1}\) through the Acer extruder. The initial water content of the extruded mix was 37.5%.

- Water content before extrusion;
- Water content after extrusion.

These differences are, however, small compared to the variation observed in the viable counts of *E. coli* found in the different samples. Survival in the first sample was 80.1 ±
31.4%, whereas in the last sample it was $3.9 \pm 3.8\%$.

The correlation coefficient, $r$, between viable count and extrudate water content (Fig. 3.9) was calculated as 0.509, and it was shown with a two-tailed $t$-test that the correlation between viable count and extrudate water content was not significant at the 0.05 % significance level. It can also be seen how a small variation in extrudate water content greatly altered the viable count of the extrudate. For example, an increase in water content from 40 to 45 % doubled the viable count.

Fig. 3.9 Correlation between survival of *Escherichia coli* (expressed as percentage of count after mixing) and *extrudate water content*, during extrusion at 50 mm min$^{-1}$ through the Acer extruder ($r = 0.509, P < 0.05$).
3.3.4 Extrusion pressure

*a. Measurements obtained using the Acer 2000 capillary rheometer*

During extrusion using the Acer 2000 Capillary Rheometer, the extrusion pressure was measured accurately via a pressure sensor at pre-set time intervals corresponding to each sample of extrudate collected. This data was used to examine the effect of pressure during extrusion on *E. coli* survival. Statistical analysis revealed a strong negative correlation between bacterial survival and extrusion pressure (correlation coefficient, $r = -0.76$, $P < 0.05$, Fig. 3.10) suggesting that survival decreased with increase in extrusion pressure. Since Harrison (1982) observed little increase in temperature during the whole extrusion process, characterising it as an isothermic process, it would appear that the reduction in viability seen after extrusion in previous sections (3.2.2, 3.2.3, 3.2.4) is mainly due to the effect of extrusion pressure.

![Graph showing the correlation between survival of *Escherichia coli* and extrusion pressure](image)

**Fig. 3.10 Correlation between survival of *Escherichia coli* (expressed as percentage of count after mixing) and extrusion pressure, during extrusion at 50 mm min$^{-1}$ through the Acer extruder ($r = -0.760$, $P < 0.05$).**
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b. Measurements obtained using the Lloyds MX50 materials testing machine

From the experiments conducted using the Acer 2000 capillary rheometer, which measures extrusion pressure at pre-set time intervals via a pressure sensor, it was shown that the survival of *Escherichia coli* is dependent on the pressure under which the pellet formulation is extruded (section 3.3.4 and Fig. 3.10). It is also possible to calculate extrusion pressures for these experiments using the ram extruder operated via a Lloyds MX50 materials testing machine. The following formula applies:

\[ P = \frac{F}{A} \]  \hspace{1cm} (eq. 3.1)

where:  
- \( P \) is the extrusion pressure during steady state flow, i.e. the phase during which extrudate is being produced (section 2.3.1.3 Materials and Methods),
- \( F \) is the force required to extrude material during steady state flow, and
- \( A \) is the area of the die.

The force, \( F \), required to extrude material during steady state flow was measured from the force / displacement profiles obtained during extrusion. The area of the die, \( A \), was calculated as follows:

\[ A = \pi r^2 \]  \hspace{1cm} (eq. 3.2)

where  
- \( \pi \) is constant and equal to 3.141, and
- \( r \) is the radius of the die.

Extrusion pressure data calculated as described above for batches of formulation mix inoculated with *E. coli* and extruded using the Lloyds MX50 materials testing machine were correlated with *E. coli* survival, using correlation analysis. The calculated correlation coefficient showed a non significant negative correlation \( r = -0.26, P < 0.05 \), Fig. 3.11).
Fig. 3.11 Absence of correlation between survival of *Escherichia coli* (expressed as a percentage of count after mixing) and extrusion pressure calculated from the force / displacement profiles, during extrusion at 200 mm min$^{-1}$ through the ram extruder operated via a LLOYDS MX50 materials testing machine ($r = -0.26, P < 0.05$).

However, in similar experiments the correlation coefficient between bacterial survival and data obtained from the pressure sensor of the Acer 2000 was $r = -0.76$ (Fig. 3.10). This difference in correlation obtained from the two pressure measuring techniques suggests that the assumption made in calculating data from the Lloyds MX50 materials testing machine, that the majority of extrudate is produced during steady state, is incorrect. The extrudate produced at the start of the extrusion process, when the force is less than that reached at steady state, and the extrudate produced at the end of the extrusion process, when the force is greater than that of steady state, will have higher and lower viable counts of *E. coli* respectively, than the extrudate produced at steady state. These survival differences and the experimental error in measuring the extrusion force during steady state from the force / displacement profiles are responsible for the inaccurate low
correlation coefficient of the data produced by the Lloyds MX50 materials testing machine.

### 3.3.5 Shear stress

The term shear stress can be explained if a fluid flowing in a straight cylinder of radius \( R \) and length \( L \) with a pressure between the two ends of the cylinder \( \Delta P \) can be imagined (Fig. 3.11). If it is assumed that the column of fluid of radius \( r \) is in laminar flow, it can be said that the applied force tending to move the liquid \( F_A \) results from the pressure difference between the two ends of the cylinder acting over the whole cross sectional area of the column i.e.

\[
F_A = \Delta P (\pi r^2) \tag{eq. 3.3}
\]

where:
- \( F_A \) is the force applied to move the liquid,
- \( \Delta P \) is the pressure difference between the two ends of the cylinder,
- \( \pi \) is constant and equal to 3.141, and
- \( r \) is the radius of column of fluid.

The force will be balanced by the viscous force tending to retard the fluid movement \( F_v \) which is due to shear stress, acting over the surface area of the column, i.e.

\[
F_v = \tau (2 \pi r L) \tag{eq. 3.4}
\]

where:
- \( F_v \) is the force tending to retard the fluid movement,
- \( \tau \) is the shear stress,
- \( \pi \) is constant and equal to 3.141,
- \( r \) is the radius of column of fluid, and
- \( L \) is the length of the cylinder.

Combining these equations gives the basic equation of capillary rheometry, which states that the shear stress is directly proportional to the distance from the centre of the capillary \( r \), and to the pressure gradient along the capillary \( \Delta P \), i.e.

\[
\tau = \frac{\Delta P r}{2 L} \tag{eq. 3.5}
\]
where: \( \tau \) is the shear stress, 
\( \Delta P \) is the pressure difference between the two ends of the cylinder, 
\( r \) is the radius of column of fluid, and 
\( L \) is the length of the cylinder.

Blair (1989) examined bacterial survival during extrusion using \textit{E. clocae} as the model inoculating organism and attributed bacterial kill to shear stress. The shear stress during extrusion of the mixes inoculated with \textit{Escherichia coli} and extruded using the Lloyds MX50 materials testing machine can be calculated by applying a simplified version of equation 3.5 which assumes that the pressure falls to atmospheric at the exit of the capillary, so it becomes:

\[
\tau = \frac{P r}{2L} 
\]  
(\text{eq. 3.6})

where: \( P \) is the extrusion pressure during steady state flow, 
\( r \) is the radius of the die, and 
\( L \) is the length of the die.

Correlation analysis between shear stress and \textit{E. coli} viability survival, expressed as per cent count of mixing survivors, showed a non significant negative correlation \((r = -0.455, P < 0.05, \text{Fig. 3.13})\). This is due to inaccurate pressure measurements (as shown in section 3.3.5b) used to calculate shear stress. Therefore, extrusion force data read from the force / displacement profiles and subsequently calculating extrusion pressure and shear stress, using equations 3.1, 3.2, 3.6, cannot be used to investigate the correlation between \textit{E. coli} survival and shear stress.
Fig. 3.12 Flow of liquid through a straight cylinder of radius $R$ and length $L$. The radius of the column of fluid, $r$, the pressure difference between the two ends of the cylinder acting over the whole cross sectional area of the column $\Delta P$ and the shear stress $\tau$ are presented.
Fig. 3.13 Absence of correlation between survival of *E. coli* (expressed as percentage of count after mixing) and apparent shear stress calculated from the force/displacement profiles, during extrusion at 200 mm min\(^{-1}\) through the ram extruder operated via a Lloyds MX50 materials testing machine (*r* = 0.455, *P* < 0.05).
3.4 Survival of probiotic bacteria during pellet production

In sections 3.2.2 and 3.2.3 the model Gram positive organisms *Bacillus subtilis* and *Staphylococcus saprophyticus* were shown to survive the extrusion-spheronisation process better than the model Gram-negative *Escherichia coli* (section 3.2.4). It was therefore anticipated that when the Gram positive probiotic bacteria *Lactobacillus acidophilus* and *Bifidobacterium longum* were introduced into pellets, their survival levels would be similar to those of the Gram-positive model organisms.

Mixes, formulated as for the model bacteria, but now containing either *Lactobacillus acidophilus* or *Bifidobacterium longum* were therefore prepared and subject to the extrusion-spheronisation process. No significant reduction in viable count was detected after preparation of the mixes containing either of the probiotic organisms (Figs 3.14 and 3.15). Average survival levels after extrusion were 27% and 56% for *L. acidophilus* and *B. longum* respectively, with 49% and 42% respectively surviving spheronisation (Figs 3.14 and 3.15). These relatively high survival rates were therefore as predicted for these Gram-positive rods.

However, contrary to the survival data obtained with the model Gram-positive bacteria, after air drying no viable organisms could be detected. *B. longum* is a strictly anaerobic organism and *L. acidophilus* is a facultative anaerobe. It would therefore appear that prolonged exposure to atmospheric oxygen during the air drying process was fatal to these organisms.

In an attempt to overcome the lethality of the air drying process to the anaerobic probiotic bacteria, freeze drying was investigated as an alternative drying method (*Materials and Methods* section 2.2.1.6). It was found that survival in pellets dried by this method, which is performed under vacuum and therefore in strictly anaerobic conditions, was 1.25% and 1.09% for *L. acidophilus* and *B. longum* respectively (Fig. 3.14, 3.15). These survival figures are better than no survival (limit of detection 0.02%) obtained after drying pellets containing the Gram-negative *E. coli* (Fig.3.5) and are resembling the survival levels of 16% and 13% obtained in air dried pellets containing Gram-positive *S. saprophyticus* and vegetative *B. subtilis* respectively (Figs 3.3 and 3.2).

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6 As in sections 3.2.2, 3.2.3 and 3.2.4, a one tailed, one sampled t-test was performed (P < 0.05).
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Fig. 3.14 Survival of *Lactobacillus acidophilus* after mixing, extruding at 200 mm min\(^{-1}\) through a 4 mm radius and 1 mm diameter die, spheronising, and either air or freeze drying. Results are presented as logarithmic percentages of the viable counts present in the formulations before mixing.

Fig. 3.15 Survival of *Bifidobacterium longum* after mixing, extruding at 200 mm min\(^{-1}\) through a 4 mm radius and 1 mm diameter die, spheronising, and either air or freeze drying. Results are presented as logarithmic percentages of the viable counts present in the formulation before mixing.
3.4.1 Effect of freeze drying on the viability of probiotic bacteria at various stages of the extrusion-spheronisation process

Although the freeze drying of pellets gave better survival of *Bifidobacterium longum* and *Lactobacillus acidophilus* than the conventional air drying technique (Figs. 3.14 and 3.15), survival levels of the Gram-positive anaerobic organisms were still poorer than those obtained after the air drying of pellets containing the model Gram-positive organisms, *Staphylococcus saprophyticus* and *Bacillus subtilis*. Since little is known about the conditions which affect sensitivity of formulation mixes of these probiotics to freeze drying, the resistance to freeze drying of *L. acidophilus* and *B. longum* at various stages of the pellet production process was determined.

Survival data in this section are expressed as percentages of the viable counts before drying. Survival after freeze drying the initial washed suspensions of *L. acidophilus* and *B. longum* were 0.12% and 2.99% respectively. When freeze drying was performed on the formulation mix, 0.84% and 3.02%, respectively survived, with 0.26% and 1.58% respectively still viable after freeze drying extrudates. Survival after the freeze drying of pellets was found to be 1.09% and 1.49% for *L. acidophilus* and *B. longum*, respectively.

It was shown with ANOVA that both probiotic organisms were equally sensitive to freeze drying whether they were in aqueous suspension, mix, extrudate or pellets. The calculated value of $F$ on the survival data of *L. acidophilus* showed the differences to be insignificant\(^7\). A similar comparison of the data obtained with the various samples of *B. longum*, again showed the differences to be insignificant\(^8\). Thus, sensitivity of the probiotic bacteria *L. acidophilus* and *B. longum* to freeze drying was not affected by sample form, i.e. whether the probiotics were in aqueous suspension, formulation mix, extrudate or pellets.

\(^7\) Analysis of variance (ANOVA) was used as a significance test because more than two sample means were compared ($P < 0.05$).

\(^8\) As for *L. acidophilus*, ANOVA was used as a significance test ($P < 0.05$).
In addition, survival of *L. acidophilus* and *B. longum* in freeze dried pellets was better than survival after air drying, where no viable probiotic cells could be detected (limit of detection 0.02 %), although statistically those differences were not significant⁹.

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⁹ Both an increase and a decrease in survival of bacteria were examined when the effects of freeze and air drying were compared, thus a two-tailed *t*-test was performed. The variances of the means were unequal, so the *t*-test was two sampled (*P* < 0.05).
Fig. 3.16 Survival of *Lactobacillus acidophilus* in samples of “washed” broth culture, formulation mix, extrudate strands and pellets after freeze drying. Statistical analysis (ANOVA, *P* < 0.05) revealed that the survival loss was not affected by sample form. The 100 % value represents the viable count of each sample before freeze drying.

Fig. 3.17 Survival of *Bifidobacterium longum* in samples of “washed” broth culture, formulation mix, extrudate strands and pellets after freeze drying. Statistical analysis (ANOVA, *P* < 0.05) revealed that the survival loss was not affected by sample form. The 100 % value represents the viable count of each sample before freeze drying.
3.4.2 Bacterial loss after inoculum preparation

In order to increase the number of viable probiotic bacteria in the final pellets, it would be necessary to increase the total number of organisms initially incorporated into the mix. However, the laboratory facilities did not allow for incubation and centrifugation of broth volumes larger than 500 ml. The possibility that bacteria were being lost during the washing process was therefore investigated since, if this did occur, it might be possible to increase the initial number of bacteria by modification of the washing process.

The mean viable counts of “washed” suspensions were therefore compared to the mean viable counts of the respective unwashed overnight culture (as described in Material & Methods, section 2.2.3). The mean viable counts of “washed” suspensions expressed as percentages of the mean count in the respective overnight broth cultures, are presented in Fig. 3.18 for *L. acidophilus* and 3.19 for *B. longum*.

Statistical analysis\(^\text{10}\) revealed that the reduction in the viable count of *L. acidophilus* after “washing” the overnight broth culture was not significant. Similar experiments with cultures of *B. longum* showed no loss of viable cells. In fact, the mean count after washing the cultures (119 %) was slightly higher than the mean count of the unwashed overnight broth cultures. However, this difference was shown to be insignificant by a one-tailed t-test (*P*<0.05). It would appear that the loss of *L. acidophilus* organisms occurred at the stage of pouring off the supernatant after centrifugation, some cells remained in the suspension and were discarded.

\(^{10}\)As in previous sections a one tailed, one sampled t-test was performed (*P* <0.05).
Fig. 3.18 & 3.19 Comparison of the viable counts of washed and unwashed overnight broth cultures of *Lactobacillus acidophilus* (Fig. 3.18) overnight and *Bifidobacterium longum* (Fig 3.19). The organisms were grown in de Man Rogosa and Sharpe (MRS) broth and Wilkins-Chalgren (WC) broth respectively. The viable counts of these broth cultures were compared with the counts of the “washed” suspensions prepared from them. A one tailed, one sampled t-test revealed that the differences in counts between the broth and the “washed” cultures were not significant.
3.4.3 Alternative pellet formulations for *Bifidobacterium longum*

Although freeze drying of the pellets produced highly significant reductions in the viable counts of *B. longum* and *L. acidophilus* greater than 50% of the viability loss occurred at the extrusion stage of pellet production (Figs 3.14 and 3.15). Results in section 3.3.4 underline the major influence extrusion pressure has on loss of viability. It was therefore predicted that if the extrusion force could be reduced, a higher proportion of bacteria might survive the extrusion process.

Boutell (1995) found that when glyceryl monostearate was incorporated into pellet formulations it increased the plasticity of a barium sulphate / Avicel mix which subsequently required less pressure for extrusion to take place. It was therefore decided to measure the extrusion force and extrusion pressure (section 3.3.3) of formulation mixes in which glyceryl monostearate had been substituted for lactose.

Glucose is often substituted for lactose in tablet formulations. The extrusion forces and pressures were therefore determined for mixes containing glucose rather than lactose to see if the forces would be less.

Also, since it is logical to deliver bifidobacterial nutrients together with *B. longum*, preparations containing fructooligosaccharides were also tested. Fructooligosaccarides have been found by Yazawa *et al* (1978) and Okada *et al* (1984) to be the main energy source of Bifidobacteria. Fructooligosaccarides after administration would provide nutritional support to the bifidobacteria delivered and to the existing bifidobacteria of the gastrointestinal tract if any. When Okada *et al* (1978) fed volunteers with 8 gram fructooligosaccharides per day for 8 days, Bifidobacteria formed a predominant population in the intestinal flora and growth of pathogenic *Clostridium perfingens* was strongly inhibited. Therefore, two formulations were tried containing standard Raftiline® and high performance Raftiline®.

To facilitate comparison of formulations, the ratio of Avicel and other powder ingredient of the formulations was kept constant. Consequently, due to differences in water solubility of the powders, the amount of water in the formulations had to be varied so that satisfactory pellets were produced (Table 3.1). An exception to this was formulation E, where the amount of glyceryl monostearate had to be increased, because a mix of equal parts of Avicel and glyceryl monostearate produced a very watery mix with no plasticity.
Miyake et al (1973), Malinowski et al (1975) and Boutell (1995) have all stressed that the amount of water added to a pellet formulation is critical and must be strictly controlled. Under-wetted material is found to feed poorly into the extruder and results in excessive pressure and friction on extrusion. It also results in the production of large quantities of fines in the spheroniser, and spheronisation is often incomplete (Fielden 1987). Material which is over-wet results in extrudate which is sticky and so tends to adhere to itself on collection and to agglomerate on spheronisation. Unfortunately formulations with good extrusion characteristics do not necessarily produce the best pellets (Newton 1996).

The extrusion forces during steady state flow were recorded when these alternative formulations were extruded using the Lloyds MX50 Materials Testing Machine through a die of 4 mm length and 0.5 mm radius at 200 mm min\(^{-1}\) speed in triplicate. The steady state extrusion force determined for the formulation mix containing lactose (E in Table 3.2), which had been used in all previous experiments, was 3.17 kN. However, unexpectedly, steady state extrusion forces were found to be higher, than for the formulation containing lactose for all new formulations (Table 3.1):

<table>
<thead>
<tr>
<th>formulation</th>
<th>ingredient and quantity</th>
<th>quantity of Avicel</th>
<th>quantity of water (% of formulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A:</td>
<td>25 g Raftiline (standard)</td>
<td>25 g</td>
<td>22 g (30.5 %)</td>
</tr>
<tr>
<td>B:</td>
<td>25 g Raftiline (HP)</td>
<td>25 g</td>
<td>30 g (37.5 %)</td>
</tr>
<tr>
<td>C:</td>
<td>25 g Glucose</td>
<td>25 g</td>
<td>15 g (23.1 %)</td>
</tr>
<tr>
<td>D:</td>
<td>30 g Glyceryl monostearate</td>
<td>25 g</td>
<td>15 g (21.4 %)</td>
</tr>
<tr>
<td>E:</td>
<td>25 g Lactose</td>
<td>25 g</td>
<td>30 g (37.5 %)</td>
</tr>
</tbody>
</table>

Table 3.1 Pellet formulations for *Bifidobacterium longum*

Four pellet formulations (A, B, C, D) incorporating standard Raftiline®, high performance Raftiline® (HP), glucose and glycercyl monostearate were derived as alternatives to the lactose formulation (E) used in previous experiments.
Fig. 3.20 Extrusion forces during steady state extrusion. Four formulations (A, B, C, D) incorporating standard Raftiline®, high performance Raftiline® (HP), glucose and glyceryl monostearate were compared to the lactose formulation (E) used in previous experiments. Extrusion forces were recorded during steady state flow when the mixes were extruded through a die of 4 mm length and 0.5 mm radius at 200 mm min⁻¹ speed using the Lloyds MX50 Materials Testing Machine. Extrusion forces of all mixes were higher than measured for the lactose mix (E).

Raines (1990) found that formulations containing a lower proportion of water required a higher applied force in order to undergo extrusion than those containing a higher proportion of water. In addition she noted that the steady state extrusion forces of the drier mixes were more uneven, which was also true for formulations C and D compared to A, B and E (Figs. 3.21 - 3.25). Raines explained this difference by suggesting that the wetter formulations passed through the die more easily and with better homogeneity than the drier formulations, possibly due to an increased “self lubrication” of the formulations at the die wall.

Formulations A, C and D contained less water than formulation E, 30.55, 23.1 and 21.4 % respectively compared to 37.5 %. When formulations A, C and D were extruded the steady state extrusion forces were higher than those found with the lactose formulation E (8.10, 12.00 and 4.24 kN respectively compared to 3.17 kN). Although the water content of formulations B and E was identical, the force required for extrusion of the RaftilineHP formulation was almost double that of lactose (6.92 compared to 3.17 kN). This
Chapter 3 Bacterial Survival during Extrusion - Spheronisation
difference in extrusion force may be attributed to differences in the particle size and shape of lactose and Raftiline HP. Unfortunately, so far, it is not possible to predict the extrusion force of a given formulation (Newton 1996).

Fig 3.21 Force / displacement profile of formulation A (25 g standard Raftiline®, 25 g Avicel, 22 g water). The steady state extrusion force of the formulation was 8.10 kN. Extrusion was performed using the Lloyds MX50 Materials Testing Machine through a die of 4 mm length and 0.5 mm radius at 200 mm min⁻¹ ram speed.
Chapter 3 Bacterial Survival during Extrusion - Spheronisation

Fig 3.22 Force / displacement profile of formulation B (25 g high performance Raftiline®, 25 g Avicel, 30 g water). The steady state extrusion force of the formulation was 6.92 kN. Extrusion was performed using the Lloyds MX50 Materials Testing Machine through a die of 4 mm length and 0.5 mm radius at 200 mm min⁻¹ ram speed.

Fig 3.23 Force / displacement profile of formulation C (25 g glucose, 25 g Avicel, 15 g water). The steady state extrusion force of the formulation was 12.00 kN. Extrusion was performed using the Lloyds MX50 Materials Testing Machine through a die of 4 mm length and 0.5 mm radius at 200 mm min⁻¹ ram speed.
Fig 3.24 Force / displacement profile of formulation D (30 g glyceryl monostearate, 25 g Avicel, 15 g water). The steady state extrusion force of the formulation was 4.24 kN. Extrusion was performed using the Lloyds MX50 Materials Testing Machine through a die of 4 mm length and 0.5 mm radius at 200 mm min⁻¹ ram speed.

Fig 3.25 Force / displacement profile of formulation E (25 g lactose, 25 g Avicel, 30 g water). The steady state extrusion force of the formulation was 3.17 kN. Extrusion was performed using the Lloyds MX50 Materials Testing Machine through a die of 4 mm length and 0.5 mm radius at 200 mm min⁻¹ ram speed.
3.4.4  *Bifidobacterium longum* survival in different formulations during extrusion

In section 3.3.4 it was shown that bacterial survival depends on extrusion pressure which is proportional to extrusion force (eq. 3.3). It would be expected that the higher extrusion forces of the above formulations compared to the average of 3.17 kN under the same extrusion conditions of the lactose formulation would produce higher bacterial kill. To confirm this, three batches of the Raftiline HP® formulation were inoculated with *Bifidobacterium longum*, mixed and extruded as described previously. After extrusion 31% of the initial *B. longum* inoculum were recovered compared to 56% from the lactose formulation.

A two-tailed, paired *t*-test\(^\text{11}\) showed no significant difference between those means (*P* < 0.05). However, since it was proven in section 3.3.4 that bacterial survival is dependent on extrusion pressure, the result of the *t*-test can be attributed to the high standard deviations of the survival means: 32 ± 16% survival in the Raftiline HP formulation and 56 ± 47% survival in the lactose formulation.

Therefore, the type of formulation into which the bacteria were inoculated had an indirect effect on *B. longum*: when a pellet formulation needed a greater force to be extruded, the applied pressure on the microorganism was increased, resulting in greater bacterial kill due to pressure.

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\(^{11}\) Since both a reduction or an increase in survival of *Bifidobacterium longum* is possible, the statistical test performed in this experiment has to cover both possibilities, thus the *t*-test was two-tailed. The samples were from different batches, therefore a paired test would be inappropriate. Therefore, a two tailed, two sampled *t*-test was performed (*P* < 0.05).
Fig. 3.26 Survival of *Bifidobacterium longum* during extrusion in two pellet formulations. Extrusion of the lactose formulation required a 3.17 kN extrusion force, compared to 1.92 kN required for the extrusion of the Raftiline HP formulations. Survival of *Bifidobacterium longum* was higher in the lactose formulation than in the Raftiline HP formulation.
Chapter 4:

SURVIVAL OF

LACTOBACILLUS ACIDOPHILUS

IN

SOLID SINGLE UNIT DOSAGE FORMS
Chapter 4 Survival of Lactobacillus Acidophilus in Solid Single Unit Dosage Forms

4.1 Introduction

The effects of the pellet production process on bacteria viability were described in chapter 3. Pellets were manufactured by extrusion-spheronisation, a complicated process involving four stages: mixing, extruding, spheronising and finally drying. Mixing was found to have no effect on bacteria viability, whereas each of the remaining stages killed part of the inoculated bacteria. More bacteria were killed during the final drying stage, whether it was conducted by air or freeze drying, than in any other stage of the pelletization process. Water must be removed for reasons of stability even though it is an essential component of the pellet formulation. Microcrystalline cellulose, the key ingredient of the formulation, absorbs water like a sponge and deforms plastically to allow the formation of extrudates and subsequently pellets. Therefore, all pellets produced by extrusion-spheronisation have to be dried.

Tablets and two piece capsules can be produced without the need to remove water and it was therefore next decided to investigate whether production of such single unit dosage forms containing probiotic bacteria would allow better bacterial survival. _Lactobacillus acidophilus_ was used as the test probiotic organism, since it had been found more robust than _Bifidobacterium longum_ to the physical stresses of pelletization (section 3.4).

4.2 Survival of _Lactobacillus acidophilus_ in tablets

Tablets produced by direct compression of formulations containing freeze dried _Lactobacillus acidophilus_ (section 2.3.2) mixed with either microcrystalline cellulose or lactose were produced as described in section 2.3.2.

Microcrystalline cellulose, an excipient used in pellet production (sections 2.1.1, 2.3.1.1), has good compression characteristics and could be tableted by direct compression without the need for any additives. Lactose, another excipient used in pellet production (sections 2.1.1, 2.3.1.1), required the addition of a lubricant such as 2% magnesium stearate to facilitate ejection from the die after compaction. Substitution of the BP grade of lactose, which had been used in pellet production, for “FlowLac” improved the flow characteristics of the formulation (Flemming and Mielck 1995).
A variety of pressures increasing in 50 MPa intervals between 0 and 300 MPa were employed to examine the effects of compaction on survival of \textit{L. acidophilus}.

4.2.1 Homogeneity of the formulation mix

Ten samples of 150 mg, which was equivalent to the weight of the compressed tablet, were taken from both formulation mixes and tested for \textit{Lactobacillus acidophilus} content by viable counting. The mean viable count per sample from the microcrystalline cellulose mix was $7.8 \times 10^6 \pm 3.7 \times 10^6$ and the mean viable count per sample from the lactose mix was $1.3 \times 10^8 \pm 0.5 \times 10^8$. Although the standard deviations of the means were high, they were considered acceptable compared to the standard deviations of previous experiments in chapter 3. \textit{L. acidophilus} was added to the two formulation mixes in different ways: to make up the microcrystalline cellulose formulation mix, freeze dried cells were directly mixed with microcrystalline cellulose. However, due to the limited supply of freeze dried \textit{L. acidophilus}, a washed and resuspended \textit{L. acidophilus} culture was used to make up the lactose mix. Forty millilitres of the \textit{L. acidophilus} resuspended culture were added to the lactose and disintegrant mix and the resulting suspension was subsequently freeze dried, as described in section 2.3.2. Both methods proved to be equally successful in producing a uniform tablet formulation mix. However, as mentioned above, supply of freeze dried \textit{L. acidophilus}, was limited and the higher viable counts observed in the lactose mix could not be achieved with the quantity of freeze dried \textit{L. acidophilus} available.

4.2.2 Stability of formulation mixes

Formulation mixes were sealed with parafilm and stored at room temperature. Three 500 mg samples from each formulation mix were taken at the same time every day. Viable counts were performed on the samples to check for \textit{Lactobacillus acidophilus} stability. Results are presented in Fig.4.1 for both formulation mixes. Statistical analysis revealed a strong negative correlation between \textit{L. acidophilus} viability and time in both formulation mixes (correlation coefficient $r = -0.95$ and $r = -0.89$ for microcrystalline cellulose and lactose mix respectively, $P < 0.05$).
Even though *L. acidophilus* survived one day longer in the lactose formulation mix, this was due to the fact that the mean viable count of *L. acidophilus* was higher in the lactose mix at day zero, rather than a survival difference between the two formulations. Statistical comparison of the means confirmed this, by showing no significant difference. The survival differences are due to the increased initial population of *L. acidophilus* in the lactose mix ($2.5 \times 10^8$ cfu/g) compared to the microcrystalline cellulose formulation mix ($1.4 \times 10^7$ cfu/g).

Fig. 4.1 Survival of *Lactobacillus acidophilus* in microcrystalline cellulose (♦) and lactose (■) formulation mixes over time. Results are presented on a logarithmic scale. Statistical analysis revealed a strong negative correlation between *L. acidophilus* viability and time in both formulation mixes (correlation coefficient $r = -0.95$ and $r = -0.89$ for microcrystalline cellulose and lactose mix respectively, $P < 0.05$).

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1 Both a decrease and an increase in *Lactobacillus acidophilus* viable count (from two different batches) between the two formulations were tested for significance, therefore a two tailed, two sampled $t$-test was performed ($P < 0.05$).
4.2.3 Susceptibility of *Lactobacillus acidophilus* to compaction

After compaction three tablets produced at each applied compaction pressure were tested for *Lactobacillus acidophilus* content by viable counting as described in section 2.3.6. Results are presented in Fig. 4.2 for both formulation mixes. Statistical analysis revealed a strong negative correlation between *L. acidophilus* survival and compaction pressure in both formulation mixes (correlation coefficient \( r = -0.81 \) and \( r = -0.75 \) for microcrystalline cellulose and lactose mixes respectively, \( P < 0.05 \)). No viable *L. acidophilus* could be detected in the microcrystalline cellulose formulation mix after compaction at 200 and 250 MPa, whereas in the lactose formulation mix no viable *L. acidophilus* could be detected after compaction at 250 MPa. Overall, survival of *L. acidophilus* in the lactose formulation was marginally greater than in the microcrystalline cellulose formulation. However, these survival differences were found to be non-significant.

Furthermore, the above findings are in agreement with the results of section 3.3.4, where survival of *Escherichia coli* was found to be negatively correlated to extrusion pressure, although the scale of extrusion pressure was less (1 - 9 MPa).

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2 As in section 4.2.2, a two tailed, paired t-test was performed (\( P < 0.05 \)).
Fig 4.2 Effect of compaction pressure on *Lactobacillus acidophilus* viability. *L. acidophilus* was inoculated in two tablet formulations: microcrystalline cellulose (■) and a lactose/lubricant mix (▲). Results are presented as logarithmic percentages of the viable counts present in the formulation before compaction.
4.2.4 Breaking load

The breaking loads of three tablets sampled after each applied compaction pressure from each formulation mix were measured as described in section 2.3.2.1. Results are presented in Fig.4.3 for both formulation mixes. Statistical analysis revealed a strong positive correlation between breaking load and compaction pressure in both formulation mixes (correlation coefficient $r = 0.95$ and $r = 0.96$ for microcrystalline cellulose and lactose mixes respectively, $P<0.05$).

It was observed in section 4.2.3 that survival of Lactobacillus acidophilus was higher in the lactose mix than in the microcrystalline cellulose mix, although the differences were found to be non significant. These differences may be explained by the significantly different tablet breaking load of the two formulation mixes. Breaking load is a measurement which expresses tablet strength. Tablet strength is related to the degree of plastic deformation materials undergo during compaction. Microcrystalline cellulose undergoes a great degree of plastic deformation during tablet compaction and therefore produces strong tablets. However, the degree of plastic deformation is less for lactose, weaker tablets are being produced, therefore less bacteria are 'crushed'.

\[^3\text{As in section 4.2.2, a two tailed, paired t-test was performed (}P<0.05).\]
Statistical analysis revealed a strong positive correlation between compaction pressure and breaking load ($r = 0.95$ and $r = 0.96$ for microcrystalline cellulose and lactose tablets respectively, $P < 0.05$).
4.2.5 Friabilty testing

Since compaction pressure is detrimental to bacterial viability, the tablets that had been produced by the weakest pressure and therefore, which counted the highest number of viable organisms were tested to see if they were acceptable according to their susceptibility to friability.

Tablets produced from application of the weakest compaction pressures, i.e. those compacted at 50 MPa, were tested as described in section 2.3.2.2. After 15 min rotation, no significant weight loss could be measured. After 20 min rotation mean weight loss was 0.1 % ± 0.0006 % and 0.15 % ± 0.0008 % for microcrystalline cellulose and lactose tablets respectively. These results, together with the results of the previous section, demonstrate that the physical characteristics of even the weaker tablets, were satisfactory.

4.3 Survival of \textit{Lactobacillus acidophilus} in capsules

From the previous experiments it has been shown that bacteria can be successfully incorporated into solid dosage systems such as pellets and tablets. However, the process parameters have to be selected carefully so that lethal damage to the bacteria is kept to minimum. Pressure (sections 3.3.4 and 4.2.3) and heat (chapter 3) are the physical stresses to which bacteria are most sensitive. Therefore, since two piece capsules are solid dosage forms that can be produced with minimum pressure and no heat, it was considered that they might provide an ideal solid vehicle for the administration of probiotic bacteria for colonic delivery.

In section 4.2.2 it was shown that \textit{Lactobacillus acidophilus} survived longer in a lactose tablet formulation mix. Also, it was desirable, where possible, to keep formulations identical throughout experiments so that comparison of results would be feasible. Therefore, a formulation mix of lactose/magnesium stearate containing freeze dried \textit{L. acidophilus} was prepared, exactly as described in section 2.3.2 for tablet production, and was used to investigate bacterial survival in hydroxypropyl methylcellulose (HPMC) two piece capsules.
Chapter 4 Survival of Lactobacillus Acidophilus in Solid Single Unit Dosage Forms

4.3.1 Capsule filling
Capsules were filled with 500 mg of the Lactobacillus acidophilus formulation mix at 2 N, in two steps as described in section 2.3.3.1. Twenty capsules were weighed before and after the filling. The mean empty capsule weight was 91.4 ± 1.6 mg and the mean filled capsule weight was 531.5 ± 28.1 mg. The mean encapsulated weight of the formulation mix, was therefore 440.0 ± 27.6 mg. However, 500 mg of formulation mix were accurately weighed to fill each capsule. This suggests that 59.5 ± 27.6 mg, or 11.9 ± 5.5 % of mix were lost during the capsule filing process as dust. This was indicated by powder dust present on the capsule filling area at the end of filling session. Loss of formulation mix was further confirmed when five filled capsules were found to contain only 36.25 ± 14.5 % of the nominal count of L. acidophilus filled per capsule. This represents a 63.75 ± 25.50 % loss compared to the number of cfu which were calculated to have been filled into each capsule.

The results suggest an apparent difference in the numbers of L. acidophilus calculated to have been lost by the two methods: only 11.9 ± 5.5 % of powder was lost during the weighing process, whereas there was a reduction of 63.75 ± 25.50 % in the viable count of L. acidophilus. Since the formulation mix contained a uniform distribution of L. acidophilus (section 4.2.1), it is unlikely that a proportion of L. acidophilus was killed during compaction of the powder mix prior to capsule filling. Although the pressure applied during compaction was very low (2 N), it was shown in the previous chapters that pressure has a dramatic effect on the viability of bacteria. However, since approximately one third of L. acidophilus still remained viable in the filled capsules it was concluded that such a high number would be sufficient for clinical administration. The application of a colon specific coat of ethylcellulose/amylose was therefore investigated in an attempt to manufacture a colon specific delivery system for the remaining viable L. acidophilus.
Chapter 4 Survival of Lactobacillus Acidophilus in Solid Single Unit Dosage Forms

4.3.2 Ethylcellulose coat

Before applying a colon specific coat of ethylcellulose/amyllose, a coat containing only ethylcellulose was applied as a control. This coat does not require heat to dry, so the physical stresses on the encapsulated bacteria are kept to minimum.

HPMC capsules were coated as described in section 2.3.3.2 without the application of heat. The solvent mixture (methanol and dichloromethane) in which ethylcellulose was diluted, was so volatile that 10 minutes after the application of a coat it was dry. Samples from capsule batches coated with from one to nine coats were tested for Lactobacillus acidophilus content, as described in section 2.3.6. It was shown that after the initial loss of L. acidophilus during capsule filing, there was no significant loss in viability during the application of the nine coats\(^4\) (Fig. 4.4). The total weight gain (TWG) expressed as percentage of the weight gained relative to the weight of the uncoated capsules was also recorded (Fig. 4.5) and was found to be positively correlated to the number of coat applications \((r = 0.99, P < 0.05)\).

\(^4\) More than two means were compared: mean Lactobacillus acidophilus viable count in uncoated capsules and mean L. acidophilus viable count in coated capsules. Therefore, analysis of variance (ANOVA) performed.
Fig. 4.4 Survival of encapsulated freeze dried *Lactobacillus acidophilus* in lactose formulation mix after applications of ethylcellulose coats. Results are presented as logarithmic percentages of the viable counts present in the formulation mix before capsule filling. After the initial loss of *L. acidophilus* during capsule filling there was no significant loss in viability during the application of nine coats.
Fig. 4.5 Total weight gain (TWG) of capsules expressed as percentage of the weight gain relative to the weight of the uncoated capsules after applications of ethylcellulose coats. Total weight gain was found to be positively correlated to the number of coat applications (r = 0.99, P < 0.05). Coat thickness equivalent to a TWG of 6% was achieved after two coat applications.
Chapter 4 Survival of Lactobacillus Acidophilus in Solid Single Unit Dosage Forms

4.3.3 Ethylcellulose/amylose coat applied and hot air dried

HPMC capsules were coated with a colon specific coating of ethylcellulose/amylose as described in section 2.3.3.2. Ethanol was used as solvent for this coating solution, since amylose is not soluble in either methanol nor dichloromethane, which were used in the control. Ethanol is less volatile than the solvent used in the previous solution (a mixture of methanol and dichloromethane). After each coat application, it was therefore necessary to heat the capsules at 50°C for 10 minutes, as described in section 2.3.3.2, to dry each coat. As for the ethylcellulose control coat, three capsule samples coated with 1, 2, 3 and up to 9 coats each were tested for L. acidophilus content, by viable counting. The first coating appeared to have no effect on the viability of Lactobacillus acidophilus but then the viable count decreased steadily during the coating procedure (Fig. 4.6). No viable L. acidophilus could be detected after application of the seventh coat (limit of detection 0.02%). Bacteria inoculated in these capsules were subjected to 50°C to a total of 70 minutes. By comparing these results with results obtained from the control coating experiment (Fig. 4.4) where coating was performed without heat application, it is concluded that L. acidophilus is killed by the application of heat. Although the applied heat (50°C) was not immensely greater than the temperature required for optimum growth of L. acidophilus (37°C), it did however have a profound effect on viability. That comes as no surprise as it has been known that a temperature of 45°C, is lethal to the vegetative cells of most bacteria, fungi and most viruses (Davis and Dulbecco 1980).

The total weight gain (TWG) was recorded as in the previous experiment (Fig. 4.7). The ethylcellulose/amylose coating solution was less viscous than that containing only ethylcellulose only solution, therefore the weight gained after each coat application was less with the ethylcellulose/amylose solution.

When the coated capsules were subjected to disintegration testing, as described in section 2.3.3.3, it was found that coat thicknesses equivalent or more than a TWG of 5% withstood degradation in a simulated stomach and intestinal environment. However, such capsules remained susceptible to bacterial degradation in a simulated colonic environment. A coat thickness equivalent to 5% TWG was achieved after application of 6 coats of the ethylcellulose/amylose solution (Fig. 4.7), whereas a 4.5% coat thickness
Chapter 4 Survival of Lactobacillus Acidophilus in Solid Single Unit Dosage Formulations

whereas a 4.5% coat thickness was achieved after applying just one coat of the ethylcellulose only solution (Fig. 4.5). However, the first coat only covers half the capsule body, therefore a second application is necessary, after which coat thickness was 6.0%. Survival of *L. acidophilus* after application under heat of 6 coats ethyccellulose/amylose solution was 0.175%, significantly less\(^5\) than survival after application of 2 coats without heat in the control experiment which was 37.42% (Fig. 4.5).

![Graph](image)

**Fig. 4.6 Survival of encapsulated freeze dried *Lactobacillus acidophilus* in lactose formulation mix after applications of ethylcellulose/amylose coats, which were hot air dried.** Results are presented as logarithmic percentages of the viable counts present in the formulation mix before capsule filling. No *Lactobacillus acidophilus* could be detected after the application of 7 coats.

\(^{5}\) In comparing the effects of coating with and without heat on *Lactobacillus acidophilus* survival, both an increase and a decrease in survival were tested for significance. A non paired test is performed for comparison of *L. acidophilus* means from different batches. Therefore, a two-tailed, two-sampled *t*-test was performed (*P* < 0.05).
Fig. 4.7 Total weight gain (TWG) of capsules expressed as percentage of the weight gain relative to the weight of the uncoated capsules after applications of ethylcellulose/amylose coats. Coat thickness equivalent to a TWG of 5% was achieved after six coat applications and was shown to withstand degradation in a simulated stomach and intestinal environment, whilst remaining susceptible to bacterial degradation in a simulated colonic environment.
4.4.4 Ethylcellulose/amylose coat applied without heat

To confirm that *Lactobacillus acidophilus* was killed by the heat applied during coating, an ethylcellulose/amylose coating was also applied without heat. One hour was necessary for each coat to dry without application of heat. Therefore, in some cases to coat one capsule completely a full working day was needed.

Viable counts of the encapsulated *L. acidophilus* were performed as soon as the coating process was completed. These were compared to the viable counts of the formulation to investigate the effects of the coating process on *L. acidophilus* viability. However, in stability experiments it was shown that *L. acidophilus* viability reduces every day (section 4.2.2). Therefore, for accurate comparison, a *L. acidophilus* viable count was performed on the formulation mix on the same time as each capsule viable count.

The *L. acidophilus* survival results (Fig. 4.8) show no significant reduction in viability\(^6\), as in section 4.3.2. This finding confirms that drying capsule coatings at 50° C is fatal to *L. acidophilus*.

Furthermore, these capsules were subjected to disintegration testing, as described in section 2.3.3.3. The findings were identical as with the ethylcellulose/amylose coated capsules which were hot air dried: a coat thickness of 5 % TWG and above was necessary for the capsules to withstand disintegration in simulated stomach and intestinal environments. However, they remained susceptible to degradation in the medium containing amylase, an enzyme produced by the colon’s intestinal flora.

The above results demonstrate that *L. acidophilus* can be successfully formulated in coated capsules, provided the coating is performed at room temperature.

\(^6\)As in section 4.3.2, analysis of variance (ANOVA) was performed.
Fig. 4.8 Survival of encapsulated freeze dried *Lactobacillus acidophilus* in lactose formulation mix after applications of ethylcellulose/amylose coats without heat. Results are presented as logarithmic percentages of the viable counts present in the formulation mix before capsule filling. After the initial loss of *L. acidophilus* during capsule filling there was no significant loss in viability during the application of nine coats.
CONCLUDING REMARKS
Concluding Remarks

The survival of probiotic and model organisms in pellets, tablets and capsules has been investigated in an attempt to formulate stable solid dosage forms, containing probiotic bacteria. Two new incorporation methods were investigated: direct incorporation of bacterial suspensions into pellet formulations and the freeze drying of mixtures of bacterial suspensions with tableting excipients. The mixing of freeze dried bacteria with tableting excipients, which has been reported previously was also investigated. The bioburden of the excipients was very low and did not interfere with the accuracy of the counting methods used. Therefore, there was no need to sterilise the excipients as described by previous workers.

Two factors were found to affect bacterial viability during the manufacture of solid dosage forms: pressure and dehydration.

Both model (Bacillus subtilis, Staphylococcus aureus, and Escherichia coli) and probiotic (Bifidobacterium longum, and Lactobacillus acidophilus) organisms were used. These organisms were selected so that a Gram-negative aerobic rod (E. coli), a Gram-positive aerobic rod (B. subtilis), a Gram-positive aerobic coccus (S. saprophyticus), as well as a Gram-positive, strictly anaerobic rod (B. longum) and a facultatively anaerobic Gram-positive rod (L. acidophilus) were included.

Pressure, whether applied during extrusion or compaction, and dehydration produced during drying, were lethal to all organisms. The process of spheronisation also reduced bacterial viable counts, due to the forces involved in the process.

The Gram-negative E. coli was found to be statistically significantly more sensitive to both killing factors of pressure and dehydration, than the Gram-positive organisms (B. subtilis, S. aureus, B. longum and L. acidophilus). The different survival characteristics of the Gram-positive and Gram-negative vegetative cells are probably due to differences in their cell wall structures. The peptidoglycan of the Gram-positive cell wall forms a thick layer outside the cytoplasmic membrane, whereas in Gram-negative bacteria, the peptodoglycan layer is thin and is overlaid by an outer membrane. Bacterial spores coats act as a barrier to dehydration and provide mechanical strength thus providing high protection to external stresses: the viability of spores of B. subtilis was unaffected by the processes of extrusion, spheronisation and air drying.
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The type of formulation into which the bacteria were inoculated had an indirect effect on bacterial survival: certain pellet formulations needed a greater force to be extruded. Thus the applied pressure on the microorganisms was increased, resulting in greater bacterial kill due to pressure.

Mixing bacteria with the formulation excipients produced no bacterial kill, whether the bacteria were in the normal hydrated state or freeze dried. In addition, the variation of extrusion conditions, such as ram speed and die length to radius ratio, did not influence bacterial viability. The above findings are in agreement with Blair (1989), who studied pellets produced by incorporating a peptone water suspension of *Enterobacter cloacae*. She observed that viable counts were reduced after the extrusion and the speronisation processes, but the survival of *E. cloacae* was not affected by ram speed.

Dehydration by both air and freeze drying killed significant numbers of bacteria. In the case of *E. coli*, no viable organisms could be recovered (limit of detection $10^2$ organisms per gram). Freeze drying did not kill bacteria so dramatically, it did however, reduce bacterial populations by two logarithmic cycles, regardless of the form in which they were suspended. This confirms the findings of Staab and Ely (1986) and Brennan *et al* (1986), who also observed two logarithmic cycle reductions after freeze drying distilled water suspensions of bifidobacteria and lactobacilli respectively.

In an attempt to overcome the problem encountered with the lethal effects of drying and pressure, which are a necessary part of pellet and tablet production, it was decided to formulate mixtures containing bacteria for incorporation into capsules in which these adverse effects are kept to a minimum. The success of this approach was confirmed by the encouraging results in chapter 4. Milojiević (1989) coated pellets with an ethylcellulose/amyllose coating to provide colonic specific delivery using a coating temperature of 50°C. This temperature proved lethal to bacteria, but it was shown that a colon specific coat, could be successfully applied to capsules containing *L. acidophilus* at room temperature, thus maintaining bacterial viability.

There is agreement in the literature over the number of viable cells of *L. acidophilus* needed in a probiotic supplement for it to exert a beneficial effect. The value of Gilliand *et al* (1978) of $10^9$ *L. acidophilus* viable cells daily over a period of several days is widely
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quoted and accepted. An initial inoculum of \(10^8\) organisms per gram was used in the model experiments reported in this thesis, resulting in the production of freeze dried pellets containing \(10^6\) and \(10^5\) viable cells of *L. acidophilus* and *B. longum* present per gram respectively. In tablets compressed at 50 MPa, \(10^7\) freeze dried *L. acidophilus* cells per tablet could be recovered initially. The sophisticated colonic delivery system of ethylcellulose/amylose coated-capsules contained \(10^7\) viable *L. acidophilus* per capsule. All these values are lower than the recommended \(10^9\) dosage, they do however show that the production of solid dosage forms containing probiotics is feasible. To achieve the required higher bacterial count in the end product, the starting bacterial population would have to be increased to allow for inevitable bacterial losses during manufacture.

Although the results in this thesis demonstrate successful probiotic tablet and capsule production, the microbial stability of the products was poor. No viable cells could be detected after 8 days in the microcrystalline cellulose formulation or after 9 days in the lactose formulation. No attempt was made to cryoprotect the organisms, which were washed and resuspended in sterile distilled water before freeze drying. However, various additives, referred to as cryoprotectants, have been used to increase survival of microorganisms during freeze drying and subsequent storage. Encouraging stability results were obtained by Staab and Ely (1986), who freeze dried bifidobacteria in a 12 % sucrose solution containing chopped meat. They found that the freeze dried bifidobacteria were still viable after one year. Phillips *et al* (1975) freeze dried *Lactobacillus ruminis* and *Lactobacillus vitulinus* in the MRS broth they had grown in, supplemented with 0.2 % cysteine HCl. Again, even after a year, the freeze dried cells were successfully revived. The above researchers stored their freeze dried cultures at room temperature. No mention of controlled humidity or refrigeration during storage was made. Wache and Viernstein (1996) compared the effect of refrigeration on freeze dried cultures of *Enterococcus faecium*. They found that viability was not significantly affected by storage at temperatures ranging from \(4^\circ\) C to \(25^\circ\) C.

This thesis has demonstrated the successful production of various dosage forms for the delivery of probiotic bacteria. The most likely to succeed was a coated capsule designed to deliver a mix of freeze-dried bacteria to the colon. However, before this could enter
clinical trials the problem of maintaining bacterial viability would have to be overcome. Although, many of the compounds demonstrating a cryoprotectant effect on microorganisms during freeze drying would not be suitable for consumption in human medicine, it should be possible, with time, to develop a capsule of long term storage at room temperature. This would be an interesting proposal for a future project.
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