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Encapsulation of Dried Yeast Cells as Probiotics by Tabletting

by

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Abstract

Probiotics are live microorganisms including yeast microorganisms and used for a wide range of applications such as food, health supplements and medicinal purposes. Due to the numerous health benefits exhibited by probiotics, they are becoming increasingly popular. Currently products are available in powder, sachet and capsule forms which are not always preferable to consumers; hence tablet dosage form is reviewed in this project.

The aim of this research was to develop a tablet formulation comprising of dried yeast granules supplied by Lesaffre (France) and understand the compaction properties of the granules and excipients by investigating the mechanical strength of primary particles using a compression technique. The mechanical characteristics of Microcrystalline Cellulose (MCC), Dibasic Calcium Phosphate (DCP) primary particles and individual yeast granules was conducted using a micromanipulation technique and Zwick/Roell Z030 mechanical tester, respectively. The mean rupture stress was found to be the highest for Actisaf STD granules whereas MCC particles did not cause rupture, reflecting its plastic behaviour during compression. The brittle behaviour of DCP particles was confirmed.

A formulation of probiotic tablets containing dried Milled 3441 yeast granules was optimised by initially varying the composition of the powder blend, which was further developed by sequentially increasing the ratio of Milled 3441 to powder blend to determine the optimum tablet exhibiting adequate cell viability (2 $x10^9$ CFU/ g per 3 times dosage) and tensile strength. It was found that tablets with increasing amount (%(w/w)) of yeast resulted in tablets with decreasing tensile strength highlighting the poor binding properties of dried yeast granules.

When investigating other processing conditions such as compaction speed and pressure, it was found that increasing the compaction pressure from 105 to 150 MPa resulted in tablets with increasing tensile strength (MPa), however had an adverse effect on cell viability (which decreased significantly). Speed, on the other hand showed a significant decrease in mean tensile strength as the compaction speed increased at high speeds of 60 mm min^{-1} with no effect on the cell viability. Based on the results, the decision of using a compaction pressure of 105 MPa and speed of 30 mm min^{-1} during the formulation development throughout this research, was confirmed.

Upon fitting the compression data to the Kawakita model, the graph can be used to represent the compression data reasonably well, from which the initial voidage of the powder blend in the die and the mean rupture stress of primary particles can be determined via constants *a* and *1/b*, respectively. However, to determine a correlation between primary particles and its corresponding Kawakita parameter *1/b*, a wider range of compressible excipients would need to be tested.

A formulation for dried yeast probiotic tablets has been produced which achieved the Pharmacopeia strength standards for future applications of scale-up process and animal use.

DEDICATION

This is dedicated to You.

Love Peace Faith

Acknowledgements

Firstly, my deepest gratitude is dedicated to Him.

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Last but by far not least, my gratitude reaches out to my best friends; 2 Peas in a Pod.

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NOMENCLATURE

a	Kawakita parameter
b	Kawakita parameter
D	Tablet diameter (mm)
d_{50}	Median from particle size distribution
F	Rupture force (N)
ε	Nominal strain
h_i	Initial height of powder bed
h_p	Height at compaction force
P	Measured crushing force (N)
t	Tablet thickness (mm)
w/w	Weight/weight
σ	Applied stress (MPa)

ABBREVIATIONS

CFU	Colony forming units
CI	Confidence interval
DCP	Dibasic calcium phosphate
MCC	Microcrystalline cellulose
Mg St	Magnesium Stearate
PBS	Phosphate buffered saline
RH	Relative Humidity
SEM	Scanning electron microscopy
TS	Tryptone Saline
YMA	Yeast Malt Agar

CHAPTER 1: INTRODUCTION

1.1 PROJECT TOPIC AND BACKGROUND

Probiotics are becoming increasingly popular due to their wide diversity of health benefits (Bafutto et al. 2013, Martindale 1982, Rokka et al. 2010), with bacterial products predominantly available on the market in the form of dairy products, capsules and sachets (Anal et al. 2007, Rivera-Espinoza et al. 2010). Products containing yeast are not as widely available, but are fast developing and becoming more common (Rokka et al. 2010). Tablet forms are not as readily available (compared to bacteria probiotics) but offer a wide range of advantages resulting in its popularity especially within the pharmaceutical industry primarily as supplements (Jivraj et al. 2000; Rubinstein et al. 2000). Probiotic tablets would be a favourable form for yeast probiotics, however proposes a challenge since cell viability is reduced during tabletting (Plumpton et al. 1986). Probiotic tablets require adequate strength to withstand transportation and handling, in addition to containing an adequate number of viable yeast cells to exhibit probiotic benefits on the host.

The objective of this project was to produce a rigid tablet containing dried yeast according to the specification provided by an industrial sponsor Lesaffre (Human Care and Feed Additives), France, which formed guidelines for testing formulations developed in this project. The specification was to produce tablets exhibiting a tensile strength of at least 1 MPa and a cell viability of at least 2 x 10^9 colony forming units (CFU) in 3 dosages of 1 g tablets, which is seen to be challenging as it is known that during compaction at high pressure, required to make strong tablets, the viability is significantly reduced hindering the probiotic health benefit (Plumpton et al. 1986). Tablets of 1 g fill weight were produced

based on the specification requirements given by Lesaffre (Human Care and Feed Additives). The inclusion of dried yeast cells into a tablet dosage form presents a challenge due to the sensitivity of yeast cells during the compression process. Cells may be damaged due to the interaction of yeast granules and excipient particles whereby the yeast granules are physically exposed to mechanical stress (Plumpton et al. 1986). Some excipients, depending on their compressibility, may generate tablets with different pore sizes, which if are large enough to accommodate the yeast granules, will cause less damage. Therefore, the mechanical strength properties of primary particles of yeast granules and pharmaceutical powder excipients were investigated, as an attempt to produce rigid tablets at low compaction pressures, hence the use of micromanipulation, which is a novel technique with regards to yeast granules and these excipients. Micromanipulation involves the compression of a single particle between two flat surfaces (Mashmoushy et al. 1998). The force being imposed on a particle during compression is measured by a force transducer and the distance between the parallel surfaces is the particle deformation (Thomas et al. 2000). From this, a force deformation curve for the particle can be obtained and particle properties such as elasticity deduced. The behaviour of particles under load during the tabletting process is widely researched (Tye et al. 2004, Denny 2002) with the Kawakita model being commonly used to characterise the compaction behaviour of excipients, identifying the initial voidage of powder bed and the strength of primary particles (Kawakita 1970). This approach allows a better understanding to be reached of the compaction properties exhibited by primary particles, and mixtures of powder excipients. As a result, the industrial challenge of producing a rigid tablet then digressed to an academic challenge of understanding the compression behaviour of primary particles during tablet compaction.

In order to produce a tablet dosage form with acceptable mechanical properties and cell viability, the formulation i.e. the selection of excipients such as the binder and filler, is also of high importance. The choice of excipients and formulation composition serves two purposes: one, to produce rigid tablets at low pressures. The second, acting as a barrier against external environmental factors such as oxygen, light and temperature which could result in varying storage stability. Typical stable storage timelines for probiotic supplements is 12 to 18 months. The mechanical properties of tablets are influenced by the role of each excipient, as well as its mechanism of compaction under pressure (Jivraj et al. 2000, Yap et al. 2008).

Upon the selection of lead formulations meeting the specification, tablets may then be tested to determine their performance for consumer use. These tests include friability, dissolution and storage stability. It is important to mention that during the formulation development stages, the tensile strength (Fell and Newton 1970) was used as a surrogate for mechanical properties and a friability test was then conducted on the selected final lead formulations, because tablets must have adequate ability to withstand handing (Fell and Newton 1970, European Pharmacopeia 5.0).

1.2 OBJECTIVES

The main objectives of this research were:

- To determine the mechanical strength of selected powder excipients and yeast granules using a micromanipulation technique and Zwick/Roell, respectively. This is used to:
    - produce tablets of primary, binary and tertiary mixtures of powder excipients and yeast granules and;
    - investigate if the mechanical strength of single particles can be related to their compaction behaviour.
- To optimise a formulation to result in tablets with a guideline tensile strength of 1 MPa and cell viability of 2 x 10^9 CFU in three dosages based on 1 g tablets given by Lesaffre (Human Care and Feed Additives), France.
- To determine if tablets of the lead formulations selected pass the Ph. Eur. (5.0) tests (such as friability, storage stability and dissolution) to ensure the final product is fit for consumer use.

1.3 THESIS OUTLINE

The organisation of the chapters in this thesis along with short summaries is given below:

Chapter 2 *Literature review:* an introduction to probiotics, their benefits and the niche for tablet dosage forms are discussed, which inspired this work. Characterisation of the behaviour of particles during tablet compaction is also summarised.

Chapter 3 *Materials and methods:* the materials and methods used to produce the tablets are introduced, and the techniques used to test the tablets for various properties are described.

Chapter 4 *Predicting the compaction behaviour of powders from their primary particle properties:* the mechanical properties of single primary particles and yeast granules were determined and related to the compaction behaviour of various compositions of tablets.

Chapter 5 *Formulation of probiotic tablets containing yeast cells in granules - effects on tensile strength and cell viability:* the best formulation with respect to the proportions of excipients and yeast granules was identified. Specific industrial granules supplied by Lesaffre, France (Milled 3441) were used for this research. In addition, experiments to determine the optimum compaction speed and force, to result in tablets with the highest tensile strength and cell viability, are outlined.

Chapter 6 *The application of a leading formulation:* a protocol formulation of powder excipients developed in Chapter 5 was tested to see if it can be applied to yeast granules of

various coating materials. Lead formulations are defined and identified on which additional tests such as dissolution, friability and storage stability were conducted.

Chapter 7 *Conclusions:* the overall conclusions of the project along with future recommendations are presented.

CHAPTER 2: LITERATURE REVIEW

In this chapter, a brief introduction to the benefits provided by probiotics for both bacterial and yeast strains are outlined, highlighting the demand for such products from the food and pharmaceutical industry. The advantages of tablet dosage forms and the commonly used excipients are also described. Details of the tablet process are given and the packing behaviour of particles during compaction is described by introducing the commonly used Kawakita model (Kawakita and Luddle 1970). Finally, some of the typical tests conducted on the tablet dosage form to ensure the product is suitable for consumers, are explored.

2.1 Introduction to probiotics and their benefits

The Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) have defined probiotics as the following: "Live microorganisms (bacterial or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host". Interest in probiotics is growing rapidly, with their health benefits increasing their popularity especially within the food industry (Rokka et al. 2010). There is a wide diversity of uses of probiotics which are considered below.

Generally, probiotic microorganisms have a therapeutic effect for improved immune system (by microbial metabolites, cell wall components and DNA), as well as the treatment of diarrhoea (antitoxin effects) by *Bifidobacterium breve* and *Saccharomyces boulardii*. Recently probiotics (*Lactobacilli* and *Bifidobacteria*) are also gaining more interest for anti-

cancer activity (Oelschlaeger et al. 2010). Reports have claimed that some probiotic microorganisms can be used for the treatment of lactose intolerance and indigestion too (Fuller et al. 1998). General review articles include a discussion of the use of gut micro-organisms as probiotics in clinical practice (Waikar 2013), a detailed review on health benefits of probiotics (Parvez et al. 2006) and another recent review provided by Amara et al (2013) which focuses on different strains of probiotics, types, and applications. Iqbal et al. (2014) also provides a review of the clinical uses of probiotics against numerous diseases such as *Lactobacillus acidophilus* and *Lactobacillus casei* for diabetes, *L. casei* and *S. boulardii* for hypertension, and lactic acid bacteria and *Bifidobacteria* for reducing episodes of upper respiratory tract infection.

The most commonly applied probiotic strains reported in the literature belong to bacterial *Bifidobacterium* and *Lactobacillus* genera (Rokka et al. 2010). Strains of yeast such as *Saccharomyces boulardii* and *cerevisea* have also been reported to provide health benefits, providing probiotic functionality, but are less common (Rivera-Espinosa & Gallerdo- Narvano. 2010). Table 2.1 lists some of the commonly used probiotic organisms (Saad et al. 2013).

Table 2. 1 Common microorganisms used as probiotic organisms (extracted from Saad et al. 2013)

Lactobacillus	***Bifidobacterium***	**Other lactic acid bacteria**	**Other**
L. acidophilus	*B. adolescentis*	*Enterococcus faecium*	*Escherichia coli* *strain Nissle*
L. casei	*B. animalis*	*Lactococcuslactis*	*Saccharomyces cerevisae*
L. crispatus	*B. bifidum*	*Leuconstocmesenteroides*	*S. boulardii*
L. curvatus	*B. breve*	*Pediococcusacidilactici*	
L. delbrueckii	*B. infantis*	*Streptococcus thermophilis*	
L. farciminis	*B. lactis*	*S. diacetylactis*	
L. fermentum	*B. longum*	*Streptococcus intermedius*	
L. gasseri	*B. thermophilum*		
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

2.2 Existing probiotic products

The use of probiotics for pharmaceutical applications has been widely reviewed and a list of commercially marketed probiotics (since 2002) with the corresponding microorganism present is provided by Kaur et al. (2002). Most commonly, probiotics have been incorporated into a wide variety of dairy food products such as yoghurts, cheese, milk powders, and ice cream (Anal et al. 2007), emerging on the market for more than 10 years (Saad et al. 2013). Recently, however, there has been an increasing consumer demand for non-dairy-based probiotic products resulting in probiotic organisms being incorporated into drinks and cereals, in addition to supplements in the form of tablets, capsules and freeze dried preparations such as Multibionta, Enterogermina, Reuterina, UltraLevure, Florastor (Rivera-Espinoza et al. 2010). Those probiotic products in the form of fermented milk, chewing gum, sachets and capsules show limited stability of the probiotic microorganisms (Klayraung et al. 2009). As found with bacterial microorganisms, tablets can be easily designed (with the addition of the right combination of tablet excipients) to control the transportation of the probiotic microorganisms to the epithelial mucosa of human host (Maggi et al. 2000, Klayraung et al. 2009). Because of its numerous advantages the tablet dosage form accounts for more than 80% of all pharmaceutical dosage forms administered to man (Jivraj et al. 2000). Tablets exhibit a wide range of advantages (as discussed below) yet the availability of yeast probiotic tablets is limited thus presents a gap in the market to meet consumers' needs, to treat hypertension, antibiotic associated diarrhoea and fewer relapses of inflammatory bowel disease (Saad et al. 2013).

2.3 Advantages of tablet dosage forms

Compaction into a tablet dosage form offers numerous advantages from a manufacturing, processing and patient point of view. With respect to processing and manufacturing, advantages include minimised number of operations involved in the pre-treatment of the powder mixture before tabletting, hence greatly reducing the production time and consequently the cost (Jivraj et al. 2000; Rubinstein, et al. 2000). In addition, tablets provide ease of manufacture, convenience and greater stability, compared to liquid preparations as fewer excipients are included in the formulation (Jivraj et al. 2000). This is true for those excipients that are sensitive to moisture, as no liquid components are added in to the formulation, therefore, there is less risk of chemical degradation occurring. This also coincides with fewer excipients needed in the formulation; hence fewer interaction issues are likely to arise.

Powder compaction by direct compression is the preferred technique compared to wet or dry granulation, especially for tablets containing dried yeast. Wet granulation is most unfavourable for preparation of yeast tablets because yeast cells become active almost immediately upon the manufacturing process thus resulting in a lower yeast count after tablet production (Al-Mohizea et al. 2007, Jivraj et al. 2000).

Direct compression has a disadvantage of segregation, an issue caused by the differences in particle size of excipients. However, segregation can be reduced by selecting a particle size and density of excipients that correspond to those of the active to result in a homogeneous

mix. In addition, sieving could also be used to minimise this issue (Jivraj et al. 2000, Khan and Rhodes 1973).

Overall, the popularity of tablets still remains resulting in a high demand and continues to be a well-researched area.

2.3.1 Tablet compaction of dried yeast

Although some tabletting conditions (speed, pressure and/or temperature) can have an effect on tablet properties for tablets containing drug substances, the functionality remains during compaction. However, when considering probiotics, tabletting conditions may have an effect on cell survival, hindering its functionality. These effects on viability are important as the medicinal efficacy of probiotic food products depends upon the number of viable cells per gram or milliliter of the products at the moment of consumption (Sohrabvandi et al. 2012). It is therefore essential to ensure a high survival rate of the probiotics during production as well as over the product shelf life (Cruz et al. 2010; Saxelin et al.1999, Sohrabvandi et al. 2012). From literature it has been seen that direct compression is commonly used during the compression of microorganisms for the intended use as probiotics (Al-Mohizea et al. 2007, Chan & Zhang 2002 and Graff et al. 2008). In addition, Al-Mohizea et al (2007) investigated the production of probiotic tablets produced using different techniques and found direct compression to be favorable over wet and dry granulation. However, it has been found that direct compression led to a significant decrease in the viability of freeze-dried yeast (Graff et al. 2008). This needs to be taken into account when formulating probiotic tablets. In many cases, it is crucial to produce tablets at low compaction pressures. This is true for bacterial microorganisms where the functionality can be lost at high compression pressures (Chan &

Zhang, 2002). The same was found with yeast where viability was significantly reduced during the tabletting process due to the pressures required (Plumpton et al. 1986). Therefore, for yeast probiotic tablets, highly compressible excipients are required which, at low compaction forces, produce tablets with an acceptable strength that can withstand handling during processing and transport. Thus, the cell viability must maintain and the challenge one faces with ensuring this is to minimise cell damage during compaction and develop a formulation that provides barrier properties for storage.

2.3.2 Tablet formulation and selection of excipients

A tablet formulation comprises of several components with different properties and functions, in most cases divided in to active ingredient(s) and excipients (such as fillers, disintegrants and antiadherents). A filler, which often constitutes a large proportion of the excipients, should ideally be inert, non-toxic, and non-hygroscopic, have acceptable taste and be inexpensive (Jivraj et al. 2000). The successful production of tablets exhibiting good mechanical properties depends upon the choice of excipients i.e. binder and filler and their proportions. The amount of each excipient, as well as its mechanism of compaction under pressure, influences the mechanical properties of the final tablet (Jivraj et al. 2000, Yap et al. 2008). When formulating tablets via direct compression, the choice of binder is critical as it must provide both binding functionality and powder flow-ability (Zhang et al. 2003). A review outlining the various excipients that have been used as fillers in direct compression formulations, with particular emphasis on what is expected from such excipients in terms of their functionality is provided by Jivraj et al. (2000).

2.3.3 Mechanical properties of particles

Particles are often characterised for various properties including size, shape, bulk and true density, powder flowability, compressibility and compactibility. The compaction behaviour of a tablet is dependent on the mechanical properties of individual primary particles (Yap et al. 2008). Understanding particle characterisation and their mechanical properties for tablet compaction is summarised by Jain (1999). A recent review is also provided by Amidon et al. (2009), who presents an overview focusing on physical and mechanical properties. The characterisation is split into three sections; particle size, powder characterization and compact (mechanical property) characterisation (some outlined in Table 2.2) with the intention to provide an understanding which can be applied to the design and development of solid dosage forms (Amidon et al. 2009).

Table 2. 2 Some typical tests and methods for the characterisation of particles, powders and tablets (taken from Amidon et al. 2009)

Method	Measured parameters
Particle characterisation	
Scanning electron microscopy	Size, shape, roughness, size range
Sieving	Size, size distribution
Light diffraction particle size	Quantitative size, distribution, span
Powder characterisation	
Bulk/tapped density	Bulk and tapped density, compressibility index
Compact characterisation	
Tablet compaction	Compaction pressure, solid fraction
Indentation test	Deformation pressure, elastic deformation
Tensile test	Tensile strength, compromised tensile strength
Tablet characterisation	
Tabletability	Tensile strength- solid fraction relationship
Compactibility	Tensile strength- compression pressure relationship
Compressibility	Solid fraction- compression pressure relationship
Manufacturability	Tablet crushing force- compression force relationship

For small size (µm to mm) primary particles, a micromanipulation technique can be used to determine the rupture stress and other mechanical properties. This technique involves the compression of a single particle between two parallel plates until rupture is reached. Before compression, samples are placed on a slide and then placed on a stage located beneath a probe. The probe is connected to a force transducer, which can set to move at a required speed. The load applied on the single particles is measured by a force transducer; which can be modified to obtain a different force range. The compressive displacements are calibrated by the transducer and the probe compliance from which the force imposed is simultaneously determined with the corresponding deformation from which force displacement plots can be produced as shown below (Sun and Zhang 2002).

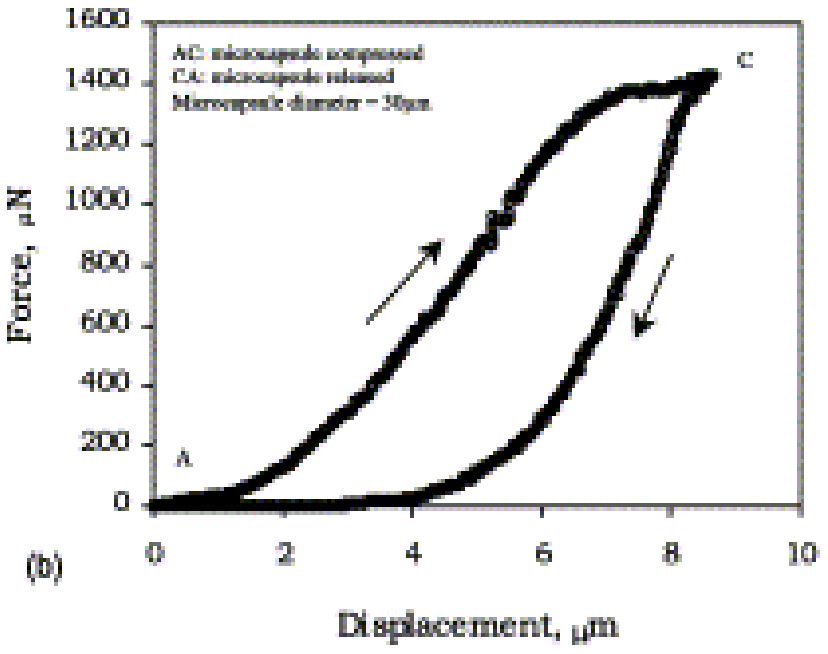


Figure 2. 1Force vs. displacement curve when single urea–formaldehyde microcapsules (microcapsule diameter = 30 µm) were compressed to a relatively large deformation and then released. A-C: microcapsule compressed, C-A: microcapsule released. The compression speed was 1 µm/s (Taken from Sun and Zhang 2002).

The technique has been used to characterise the mechanical properties of many single biological and non-biological particulate materials, including animal cells, plant cells (Thomas et al. 2000), yeast (Mashmoushy et al. 1998), bacteria (Shiu et al. 1999), latex aggregates, microcapsules (Sun & Zhang. 2002) and pharmaceutical excipients (Yap et al. 2006). The study of compressibility of dried yeast (*S cerevisiae*) has been of interest for many years (Dobbs et al. 1982), however applying this micromanipulation technique has not been conducted previously and proving novelty to this research**.** The micromanipulation of dried yeast granules supplied by Lesaffre forms a key feature of this thesis. Hence, particle characterisation allows the understanding of powder compaction properties for tableting which are vital as discussed below (Jain 1999).

2.3.4 Tablet compaction process

As a powder is compressed within a die, the compaction process can be separated into stages of rearrangement, deformation, compaction, and relaxation as described by Jivraj et al (2005). To summarise, at low compaction pressures particles are initially rearranged to sit more closely together and the powder bed porosity and volume are reduced.

As the pressure load continues, a point is reached at which all the voids are filled and no further rearrangement can occur. The volume reduction that then takes place is associated with changes in the particles, either being temporary (i.e. elastic deformation) or permanent by plastic deformation (Figure 2.2). Particles could also change by brittle fracture into smaller particles, which subsequently undergo a secondary particle rearrangement followed by plastic and /or elastic deformation.

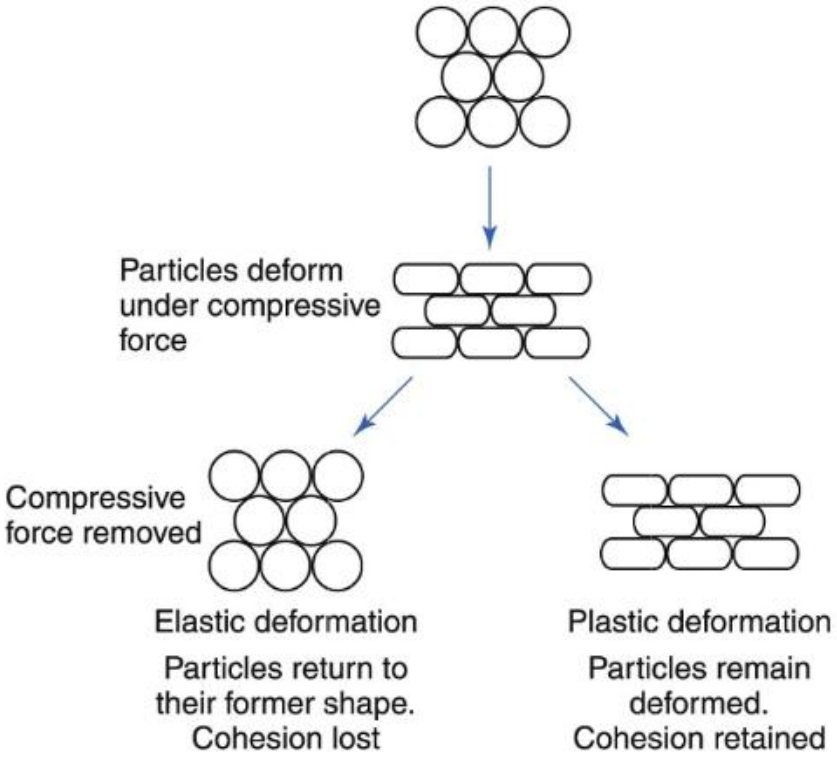


Figure 2. 2 Schematic illustration of particle deformation under load (figure taken from Alderborn, 2002)

It is important to achieve the right balance of excipients undergoing brittle fracture (such as Dibasic Calcium Phosphate) and plastic behaviour (such as Microcrystalline Cellulose) by incorporating the right quantities of each (Jivraj et al. 2000; Roberts et al. 1987). Upon the removal of load, particles (or the compact) may expand due to elastic recovery (Alderborn 2002) which must be considered since the properties of the final product can be affected.

The arrangement of particles as a result of particle deformation accounts for the porosity of tablets (Capece et al. 2014) which is of high importance since it can influence mechanical properties of tablets (Wu et al. 2007; Mattsson et al. 2001). Ryshkewitch and

Duckworth (1953) established the relationship between bulk porosity and tensile strength, stating that there is an exponential relation between these two parameters. More recently, it has also been shown that pore size influences tensile strength (Wu et al. 2007). Mattsson et al. (2001) studied the effect of different binders on the pore structure and bonding properties of tablets and found that tablets with the higher tensile strength were formed with the more deformable binder, as a result of a lower porosity. It was also found that tablet strength was lower with binders displaying elastic behavior resulting in overall higher porosity than those displaying plastic behavior (Mattsson et al. 2014). For tablets containing microorganisms for e.g. probiotic applications, it can be assumed that a tablet exhibiting a lower porosity would result in greater damage to microorganisms as a result of the packing arrangements as seen later in Chapter 5 (Section 5.2.2.2). A detailed description of the stages from primary particles to tablet for the tabletting process is provided by Hamad et al. (2010).

2.3.5 Analysis of compaction process

Powder compaction behaviour can be defined as compressibility (solid fraction vs. compression pressure), tabletability (tensile strength vs. solid fraction) and compactibility (tensile strength vs. compression pressure) as outlined by Amidon et al. 2009. A compaction equation relates some measure of the state of consolidation of a powder, such as porosity, volume (correlative volume) density, or void ratio, with a function of the compacting pressure (Denny 2002). Many models have been used to characterise the compaction behaviour of primary feed particles including Heckel (1961), Kawakita (1970) and Adams (1994). The Kawakita model represents compaction data to relate the volume reduction

(engineering strain) of a powder bed to the applied pressure (Equation 2.1). The work is summarised in the paper by Kawakita and Ludde (1970).

$$\frac{\sigma}{\varepsilon} = \frac{1}{ab} + \frac{\sigma}{a} \qquad \textbf{Equation 2.1}$$

where σ is the applied stress and ε is the nominal strain (consequential degree of volume reduction), given by equation 2.2:

$$\varepsilon = \frac{h_i - h_p}{h_i} \qquad \textbf{Equation 1.2}$$

where h_i is the initial height of the powder bed, h_p is the height at compression force and *a* and *b* are constants.

A typical nominal stress- strain curve (Figure 2.3) can be used as a basis to form the Kawakita plot.

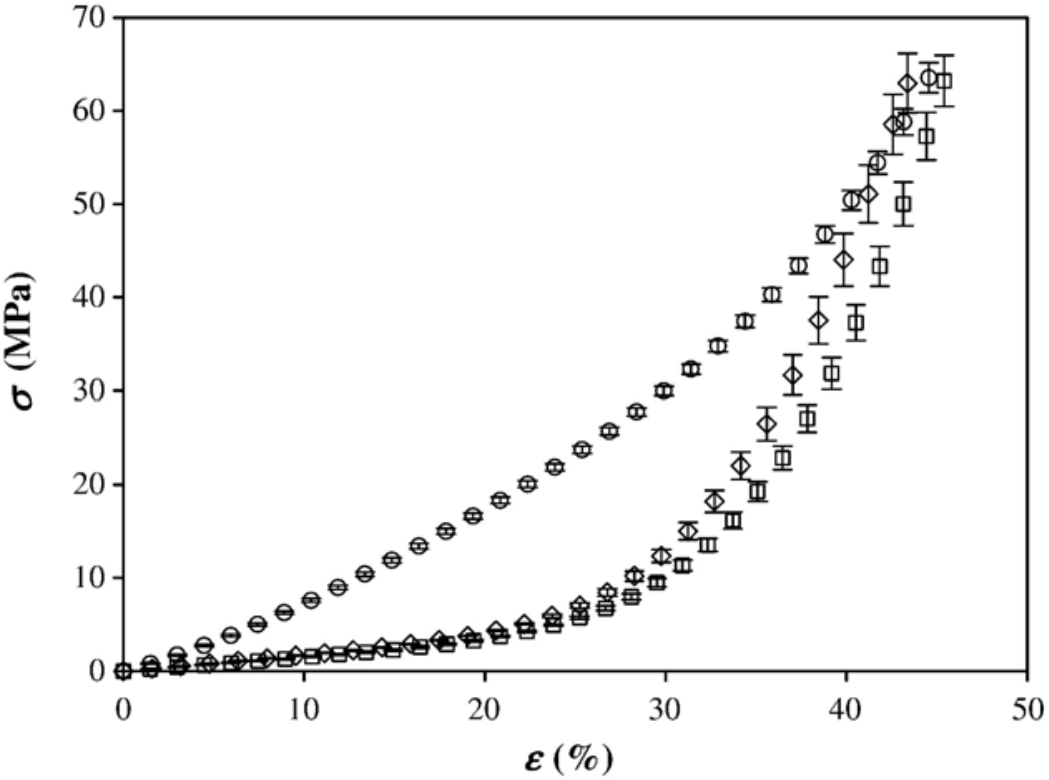


Figure 2. 3 Nominal stress–strain curve for the bulk compression of Eudragit® L100-55 (○), L100 (◇) and S100 (□) powders for an aspect ratio of 1.0 (Yap et al. 2008).

The Kawakita model predicts that there is a linear relationship between σ/ε and σ (Figure 2.4). The slope and intercept allow the constants *a* and *b* to be easily evaluated (Kawakita 1970, Yap et al. 2008). It can be shown that constant, *a*, is equal to the initial value of the porosity. However in practice, it has been found that its derived value does not always agree well with the measured value, often due to the non-linearity of the plots (Denny 2002). The constant, *b*, has the dimension of the reciprocal of stress representing the nominal stress of individual particles (Adams et al. 1994). Although most Kawakita plots show linear relationships throughout the whole range of pressures, many show curvature especially at the low pressure end. Kawakita and Ludde (1970) stated that this equation holds best for soft fluffy pharmaceutical powders.

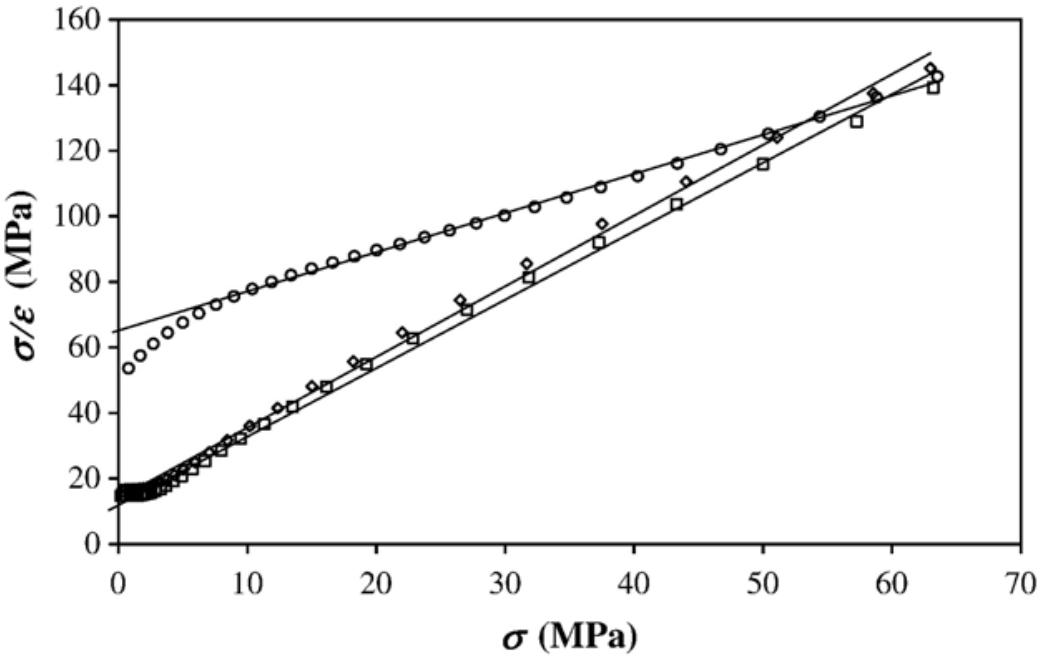


Figure 2. 4 Typical linear Kawakita plot for the bulk compression of Eudragit® L100-55 (○), L100 (◇) and S100 (□) powders for an aspect ratio of 1.0 (Yap et al. 2008)

The reason for its popularity could be its simple mathematical form, and also the fact that substantial knowledge has already been built on the basis of its use, or more importantly, that the equation is attractive because of the physical significance of the compression parameters. Supporting evidence is provided by Adams et al. (1994) who looked into the compaction of porous granules where the load–deformation curve was shown to be consistent with Kawakita's equation. The single agglomerate strengths derived from the whole bed deformation curves, agreed in value with the strengths found on individual granules and allowed a physical interpretation to be made of the *b* parameter in the Kawakita equation (Adams et al. 1994).

Nordstrom et al. (2012) proposed a protocol for the classification of powder compression characteristics, looking at mannitol and sodium chloride as the test materials. It was found that experimental data fitted the Kawakita equation over a wide pressure range with high correlation coefficients (R^2> 0.999) for the linear regression, confirming the application of this model to a wide range of excipients.

2.4 Tablet characterisation testing

Upon manufacture, tablets are subjected to bulk handling and other operations during which the bioavailability and mechanical integrity must be maintained from manufacturing to consumer (Sinka et al. 2009). The tablet tests are extensively described in the European Pharmacopeia (Ph. Eur.) 5.0 (Chapter 2.9. Pharmaceutical Technical Procedures) providing detailed methods and criteria for results to determine those tablets deemed acceptable for market. Some of the typical tests, which have also been employed in this project include *dissolution* (Ph. Eur. 5.0 Chapter 2.9.3), *uniformity of mass* (Ph. Eur. 5.0 Chapter 2.9.5), *friability of uncoated tablets* (Ph. Eur. 5.0 Chapter 2.9.7), *resistance to crushing* (Ph. Eur. 5.0 Chapter 2.9.8), and *storage stability*.

The *dissolution test* determines the release profile of an active from the solid dosage form in a liquid medium (different mediums may be used if pH has an effect). Again this test requires placing 6 tablets in 6 vessels containing 500 mL of medium, where they are stirred at 100 rpm using either a paddle or basket apparatus (Ph. Eur. 5.0 Chapter 2.9.3). A water bath is used to ensure the dissolution medium is maintained at 37 ± 0.5°C. Samples of medium are taken at time intervals and the amount of active released is plotted against time.

The *uniformity of weight test* is undertaken to ensure consistency during production. This involves weighing individually 20 tablets taken at random and not more than 2 of the individual masses should deviate from the average mass by more than the percentage deviation of 5 (for uncoated tablets with average mass of 250 mg or above).

Friability is considered whereby the tablet surfaces are damaged and/ or show evidence of lamination or breakage when subjected to mechanical shock or attrition. The test involves placing 10 tablets loosely brushed and pre-weighed into the drum which is rotated 100 times. Tablets are then re weighed. Friability is expressed as the loss of mass and is calculated as a percentage of the initial mass, by which a maximum of 1% loss of the mass of tablets is considered to be acceptable (Ph. Eur. 5.0. Chapter 2.9.7).

The strength of tablets can be determined by measuring its *resistance to crushing*, under defined conditions, the force needed to disrupt the tablets by crushing. The apparatus comprises of 2 jaws facing each other, one of which moves towards the other. A tablet is placed between the jaws and pressed until fracture is caused. The measurement is conducted on 10 tablets. Results are expressed in Newtons as the mean, minimum and maximum values of the forces measured (Ph. Eur. 5.0. Chapter 2.9.8). A lack of specification is seen here. A range or a specified value which is deemed as acceptable is not defined, hence there is a need to rely on friability tests. However, it may not always be feasible to conduct a friability test especially during formulation development, due to the large sample number required (n = 10).

Lastly, a *storage stability* study is conducted to determine how environmental factors such as temperature and humidity affect the quality of tablets physically and functionally, in order to identify the shelf life and ideal storage conditions for patients (FDA 2003). Accelerated, short term studies lasting 4, 8 and 12 weeks under extreme conditions of high temperature and humidity (e.g. 40°C and 75% RH) can be conducted. Conversely, long term studies of 1- 2 years studies can be also be conducted typically at room temperature and humidity. In some cases, where the active may be sensitive to temperature, tablets may be stored under cold conditions (4°C) to identify if storing tablets in the fridge is necessary. Results from this test are usually obtained by determining the functionality of tablets (i.e. amount of active remaining), visual defects, changes in mechanical properties and/ or changes to the dissolution profile (FDA 2003).

Overall, the quality and performance of tablets are tested in order to ensure the functionality of active has remained intact post production and the final product is fit to patient use.

2.5 Conclusion

The benefits of probiotics and their applications were introduced in this chapter. To formulate a tablet dosage form, the importance of mechanical properties of primary particles and techniques to determine them were reviewed in addition to investigating particle deformation behaviour during compaction. Furthermore, analysing the compression behaviour of particles during tabletting process, using the Kawakita model, was described.

It was concluded that there are challenges for the introduction of dried yeast in to tablet dosage forms due to cell viability loss during tabletting impairing its probiotic functionality. The mechanism causing cell viability loss during manufacture of yeast probiotic tablets needed to be determined to then investigate methods of preventing this damage, resulting in tablets with higher yeast cell number.

CHAPTER 3: MATERIALS AND METHODS

3. 1 INTRODUCTION

This chapter describes the materials and methods used to produce tablets, test the resulting tablets for their mechanical strength and cell viability properties in addition to, determine the effects of tabletting conditions on tablet properties. The investigation began with a screening process to select potential dried yeast samples, from which a formulation including excipients was optimised in order to produce tablets with desirable characteristics. The inclusion of dried yeast into a tablet form was investigated further by analysing the compaction behaviour taking into consideration the primary particles.

3. 2 YEAST SELECTION BY SCREENING

3.2.1 Materials- Yeast samples

The yeast samples in the form of granules, kindly provided by Lesaffre, Human Care and Feed Additives (France) with their detailed descriptions, are outlined in Table 3.1.

Table 3. 1 Detailed list of all yeast granule samples received from Lesaffre

The code names of the samples are those provided by Lesaffre

Yeast sample	Description
Actisaf STD	Strong, robust, has a protecting external layer around the yeast cells, dried via drum drier at 40- 42°C for 10- 20 hours. (93% dry cell weight). Wide granulometry 500-2000 µm.
Pro GI+ ms 0275	Milled yeast granules, uniform, sphere shaped, dried in a fluid bed drier, small spheres + regular particle size distribution, d_{50} = 430 µm.
Pro GI Milled 3441	Milled Actisaf yeast granules. The mill chamber was equipped with a rotating blade assembly that reduced the size of particles by cutting or impacting them with a screen. The milled particles were discharged through a 30 mesh screen (mesh size= 590 µm). The mill chamber was cooled (using a water jacket) and the size reduction made under inert gas (nitrogen). The yeast spherules passed through the mill once. d_{50}= 430 µm.

Yeast sample	Description
HR+ 2585	Granules processed with additional drying (those granules that were more water and mechanically resistant) containing 94-95% solids.
L47	Sieved Actisaf STD sample particle size between 217 and 500 µm, (strong, robust, with a protecting external layer around the yeast cells, dried in a drum drier).
7J	Dried in a fluid bed dryer (drying time: 20 - 30 min (batch)), instant active dried yeast named 7J.

3.2.2 Cell viability determined by a cell enumeration technique

In order to shortlist the yeast samples to be used for the formulation of tablets, samples were subjected to three tests starting with cell viability. This indicated the amount of yeast present and was taken to be the control value. The total number of viable yeast cells (those considered as colonisable) was determined by an enumeration technique in order to determine the number of colony forming units per gram of sample. The technique was defined in a protocol provided by Lesaffre (France) and can be found in Appendix 1. Below is an overview of this technique where all experimental work was conducted at the University of Birmingham.

3.2.2.1 Preparation of reactive agents and culture medium

Firstly, Tryptone Saline (TS) Buffer solution was produced by accurately weighing out 1 g of BactoTryptone (BD Biosciences, UK) and 8.5 g Sodium Chloride (Sigma-Aldrich, UK), which were completely dissolved in 1 L distilled water. The yeast growth medium was prepared by dissolving 16.4 g Yeast Malt Agar (YMA) (Sigma-Aldrich, UK) in 400 mL distilled water. Both mediums were autoclaved at 115°C for 15 min. The YMA medium was maintained at 48°C at which point 4 mL of oxytetracycline solution (1%) was added using a sterile pipette. Oxytetracycline solution (1%) was prepared by dissolving 1 g Oxytetracycline (Sigma-Aldrich, UK) in 100 g sterile demineralised water using a magnetic stirrer. Once fully dissolved, 2 mL of hydrochloride acid was pipetted in to this solution.

3.2.2.2 Preparation and dilutions of the sample

1 g of yeast sample was weighed and added to 100 mL TS Buffer solution, pre-warmed to 37°C. The suspension was homogenised using an UltraTurrax® homogeniser (IKA, Germany) at 10 000 rpm for 3 min. Successive decimal dilutions were prepared in distilled water (pre-warmed to 37°C), using a separate pipette for each transfer. 10-fold serial dilutions were conducted to achieve a countable colony count in the range of 30-300 (typically 10^{-6} and 10^{-7}), as described below.

3.2.2.3 Plating and incubation

0.1 mL of the required dilutions (10^{-6} and 10^{-7}) was transferred to the surface of YMA Petri plates using a 0.1 mL sterile pipette in three replicates and gently spread using the surface spread plate technique, ensuring an even distribution of yeast. The plates were incubated in a reverse position at 25°C for 3 days and colonies were enumerated manually using a counting chamber. Yeast colonies on an agar plate are shown in Figure 3.1, which were distinguishable due to their large colony size compared to smaller bacterial colonies and dust specs that may be present. As a result, colonies were clearly identified ensuring that only yeast colonies are counted. In addition, the oxytetracycline was included during the yeast malt agar medium which ensured the prevention of bacterial growth. Therefore, any agar plates which showed the growth of smaller or disordered colonies were discarded.

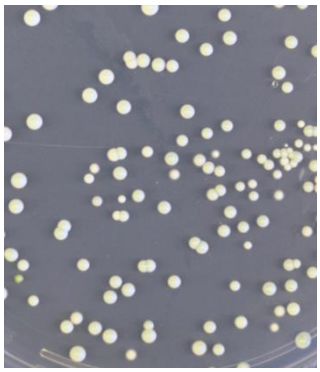


Figure 3. 1 Yeast colonies grown on yeast malt nutrient agar plate

3.2.2.4 Calculating the colony forming units (CFU)

The CFU /g yeast sample for each sample was calculated using the following equation:

$$\frac{number\ of\ colonies\ x\ dilution\ factor}{volume\ plated} \qquad \textbf{Equation 3.2}$$

In the case of determining yeast cell viability of tablets, the same equation (Equation 3.1) was applied and results were expressed as CFU /g tablet. Variability in viability between samples was seen across the range of dried yeast samples (Figure 3.2). The shortlist of selected yeast samples is discussed in Sections *3.2.4.1* and *3.2.4.2*.

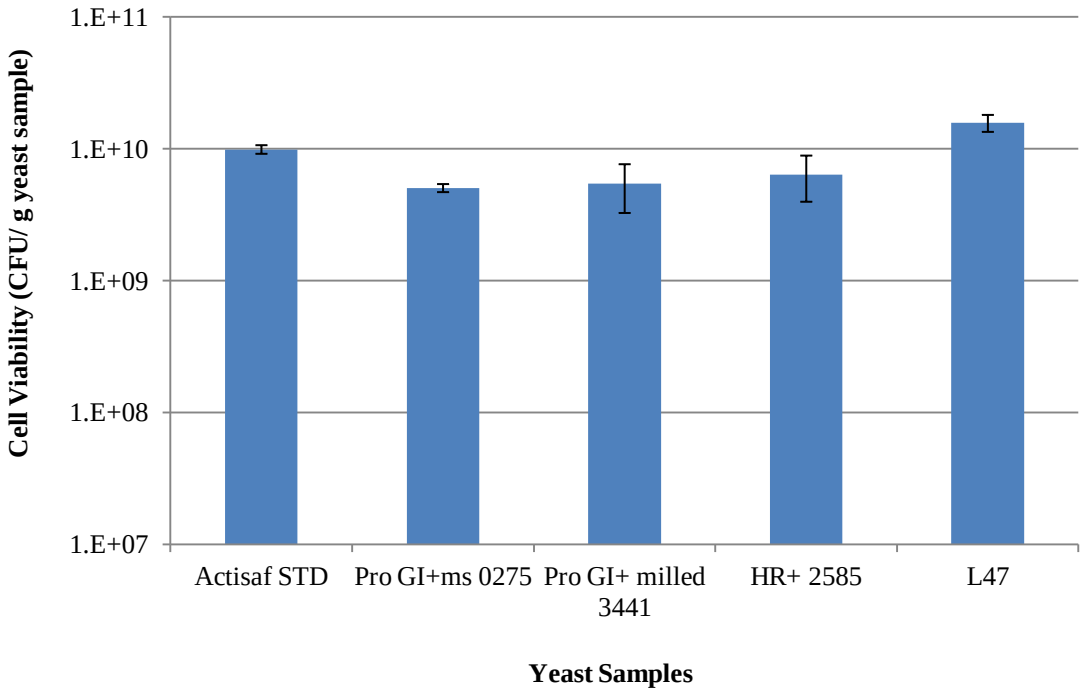


Figure 3. 2 Enumeration of total yeast living cells for all dried yeast samples supplied by Lesaffre (France). Mean ± 95% CI (n=3)

3.2.3 Tablet compaction of yeast samples

Tablets of the various yeast samples were produced as an initial screening process to determine for further testing.

3.2.3.1 Materials

The selected binder in the tablet formulation was (anhydrous) Dibasic Calcium Phosphate (anhydrous), (DCP), Sigma Aldrich, UK. The use of DCP was based on its good compaction properties as well as the calcium content it exhibits, which is advantageous for nutritional supplement products (Rowe et al. 2009). To bring the formulation to the

appropriate final weight of 1 g, a filler was incorporated: Microcrystalline Cellulose (MCC, Avicel PH102), kindly supplied by FMC Polymer, UK. The selection of MCC was based on its ranking of being one of the most useful fillers for direct compression (Shangraw et al. 1993). Magnesium Stearate (Mg St), (Sigma Aldrich) was included as a lubricant since it is known to have no interactions with DCP and MCC (Jivraj et al. 2000).

3.2.3.2 Formulation and mixing

The dried yeast was mixed with the powder excipients in batches (ranging from 10 to 100 g batch size) according to the formulation in Table 3.2 using a double cone Gardner Laboratory Mixer (Kemutec Manufacturing Division, UK) for 2 min.

Table 3. 2 Probiotic tablet formulation containing Microcrystalline Cellulose (MCC) and Dibasic Calcium Phosphate (DCP) with a constant amount of Magnesium Stearate (Mg St).

Excipient	Amount %(w/w)
Yeast sample	50
MCC	25
DCP	24.25
Mg St	0.75

3.2.3.3 Tablet preparation

Tablets were produced individually by accurately weighing 1 g powder from the bulk mixture. Powders were compressed uniaxially into tablets by direct compression using a single punch tablet press (Zwick/Roell Z030, Germany) connected to a computerized compression force analyser, under constant conditions (18 ± 0.5°C, 40 ± 10% relative humidity). The weighed formulations were filled into an Atlas 13 mm Evacuable Pellet Die (Specac, UK). All tablets had 1 g total weight and were formed at a compaction speed of 30 mm min^{-1}. The uniaxial compression tests were repeated five times; n=6 (unless stated otherwise) for each formulation, under a maximum load of 14 kN (compression pressure ca.105 MPa).

3.2.4 Tablet characterisation

3.2.4.1 Cell viability post tabletting process

Yeast cell viability was determined by counting colony forming units on yeast malt agar plates as described in Section *3.2.3.1- 3.2.3.4.* All results are expressed as mean ± 95% Confidence Interval (CI). The cell viability of probiotic yeast post tabletting was determined (Figure 3.3). Since no difference was observed across the samples, Actisaf STD was selected as it is a well established product provided by Lesaffre (France) where the production ensures reproducibility within batches.

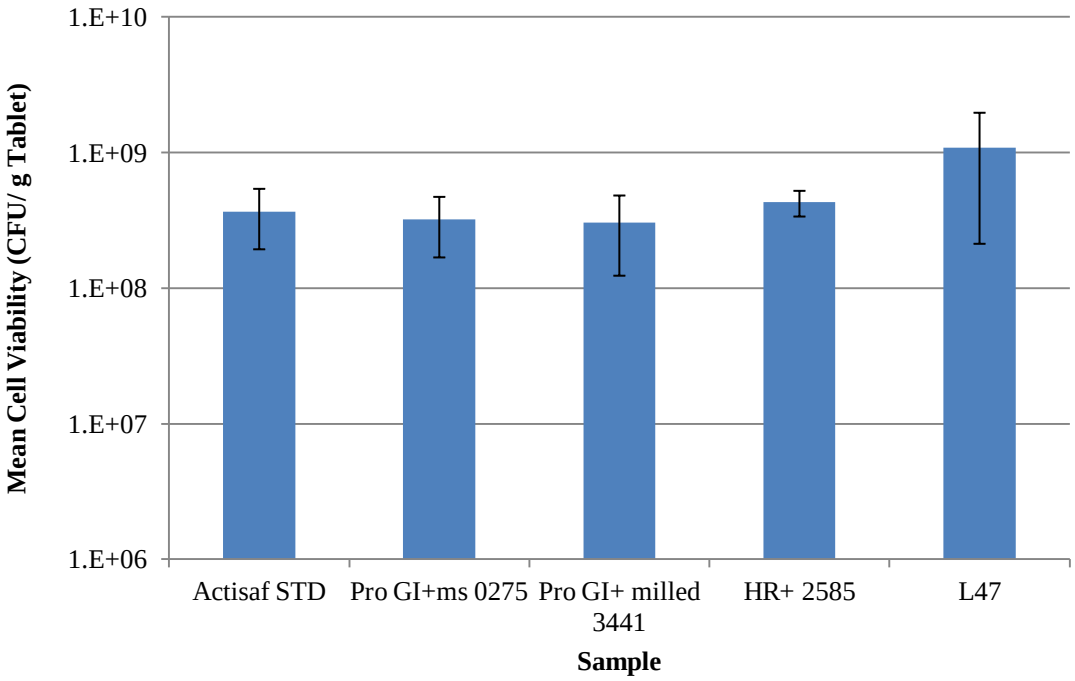


Figure 3. 3 Mean cell viability (CFU /g tablet) of probiotic tablets comprising of various yeast samples produced according to Table 3.2 using 14 kN compaction force (ca. 105 MPa compaction pressure). Mean ± 95% CI (n=3).

3.2.4.2 *Determining the tensile strength via a diametric compression test*

Tablets were tested for their mechanical strength by determining their tensile strength using the diametric compression test (Fell and Newton 1970). Tests were performed using a Zwick/Roell Z030 mechanical tester operating parallel platens at a compression speed of 5 mm min^{-1}. The force (N) required to cause failure was converted to the corresponding tensile stress σ (MPa) using the following equation (Fell and Newton 1970):

$$\sigma = \frac{2P}{\pi Dt}$$ **Equation 3.3**

where *P* is the measured crushing force (applied load), *D* the tablet diameter and *t* its thickness. Tablets for each condition were tested in triplicates. Results are expressed as mean ± 95% CI. The tablet strength of probiotic yeast post tabletting was determined (Figure 3.4) and shows the good compaction properties of Milled 3441 (resulting tablets with a mean tensile strength of 1.3 ± 0.1 MPa).

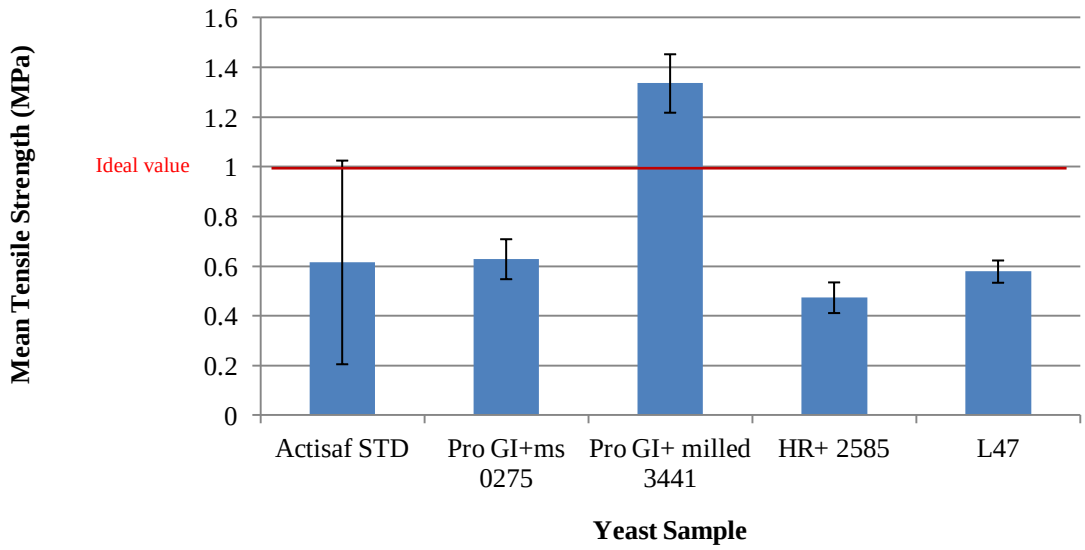


Figure 3. 4 Mean tensile strength of probiotic tablets comprising of various yeast samples produced according to Table 3.2 using 14 kN compaction force (ca. 105 MPa compaction pressure). Mean ± 95% CI (n=3).

3. 3 CHARACTERIATION OF PRIMARY PARTICLES

Individual primary particles were characterised for their size and mechanical properties. The properties of tablets comprising of single, binary and tertiary components were determined to discover if they might be predicted from the properties of single particles. Analysing compaction data and fitting stress-strain curves to empirical models were also used for the prediction as discussed in Chapter 4.

3.3.1 Particle size

3.3.1.1 Image analysis using QICPIC

A QICPIC particle size analyser (Sympatec, UK) (Figure 3.5) was used to measure the particle size distribution using a gravity disperser GRADIS/L which was combined with the vibratory feeder VIBRI/L.

Figure 3. 5 Particle size and shape analyser (Qicpic, Sympatec, UK)

The sphericity, defined by the Sympatec manual, is the ratio of the perimeter of the equivalent circle. The result is a value between 0 and 1, where the smaller the value, the more irregular the shape of the particle. All excipient particles were measured in triplicate and the d_{50} values expressed as mean ± 95% CI. Results are represented as particle size distribution graphs and sphericity graphs in Chapter 4 (Figures 4.1 to 4.4) and discussed in Section 4.2.1.1 under "Characterisation of primary particles".

3.3.1.2 Measurements using digital callipers

The size of individual Actisaf STD granules were also measured using a digital calliper (Mitutoyo, UK) as presented in Chapter 4, Table 4.1.

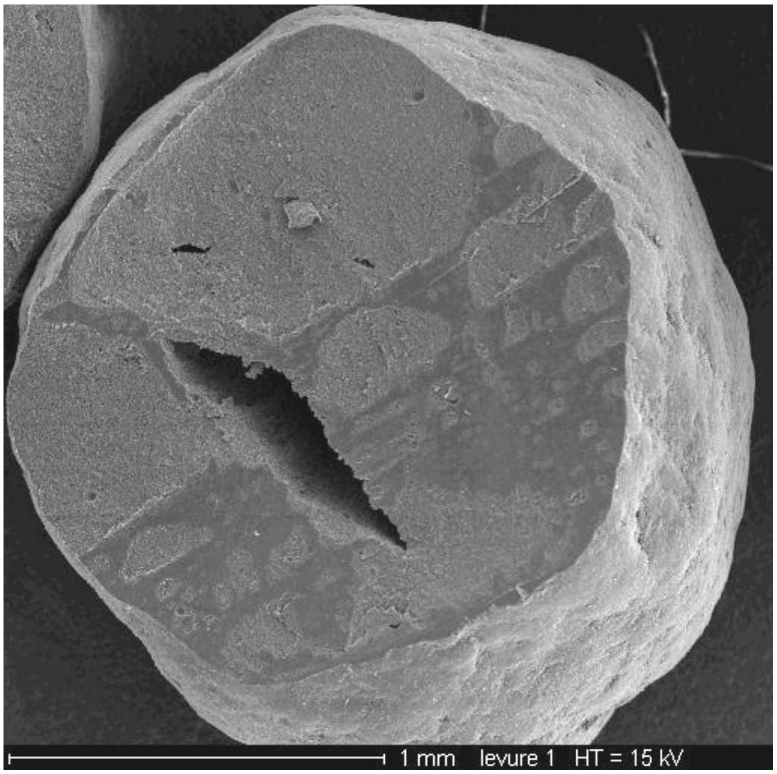
3.3.2 SEM Images

Images of excipient particles and tablets of various formulations were produced using a scanning electron microscope (SEM) by Lesaffre (France) at magnifications varying from 20 x to 10000 x. Images of sample preparation are shown in Figures 3.6a and 3.6b.

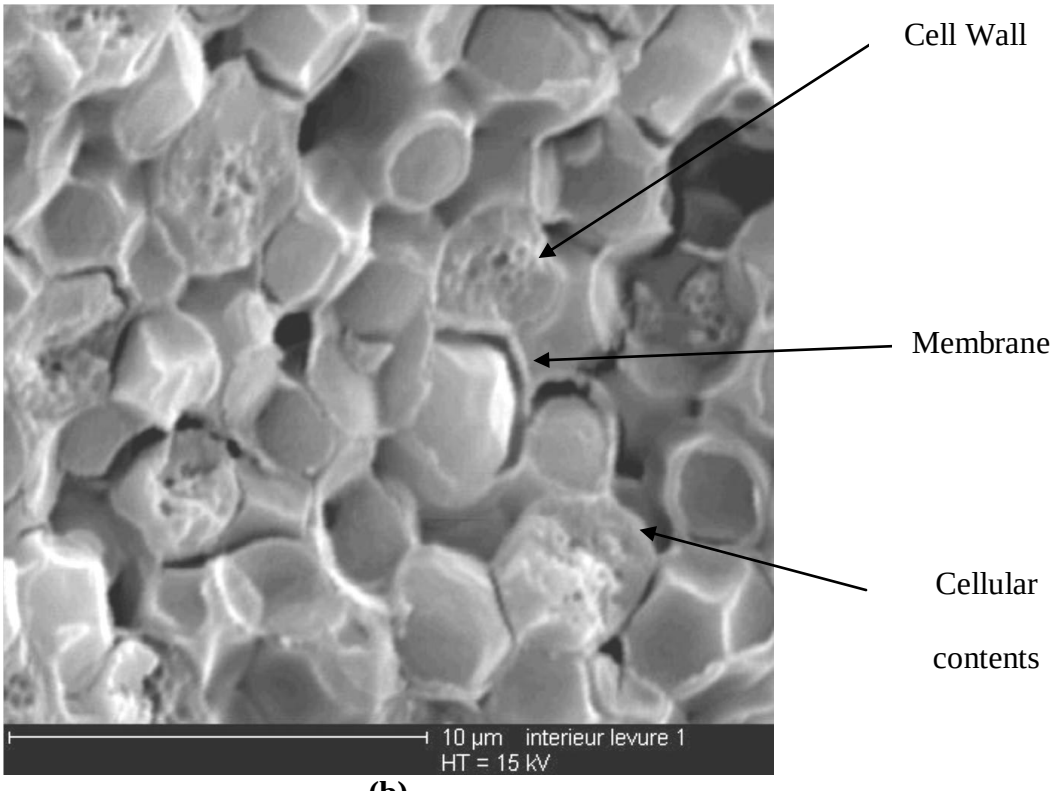
(a) **(b)**

Figure 3. 6a and 3.4b Images of samples (a) Milled 3441 granules (b) probiotic tablets containing Milled 3441 granules prepared for SEM.

SEM images of Actisaf STD granules and Milled 3441 dried yeast granules, kindly provided by Lesaffre Human Care and Feed Additives (France) are shown below in Figures 3.7 and 3.8.

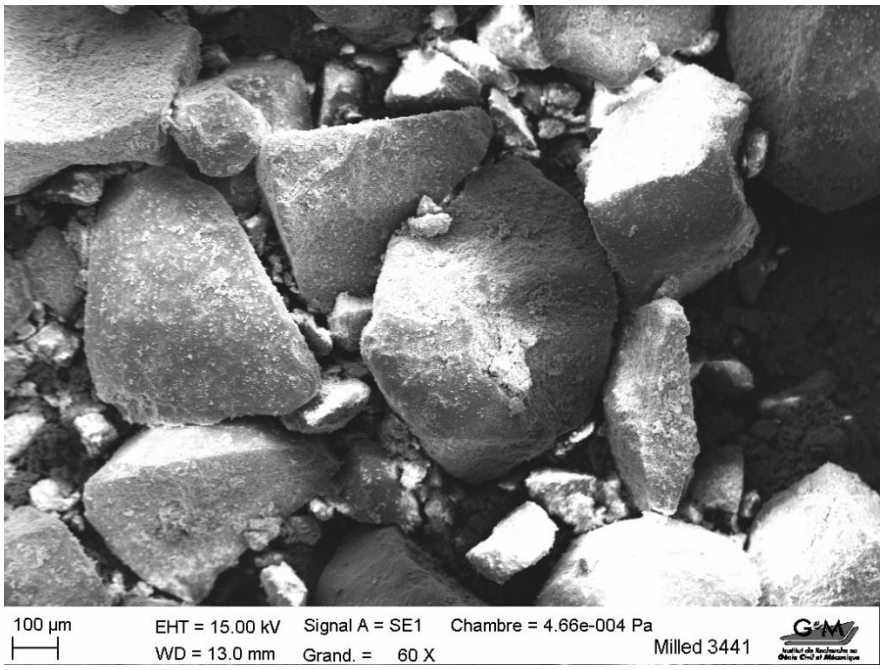


(a)

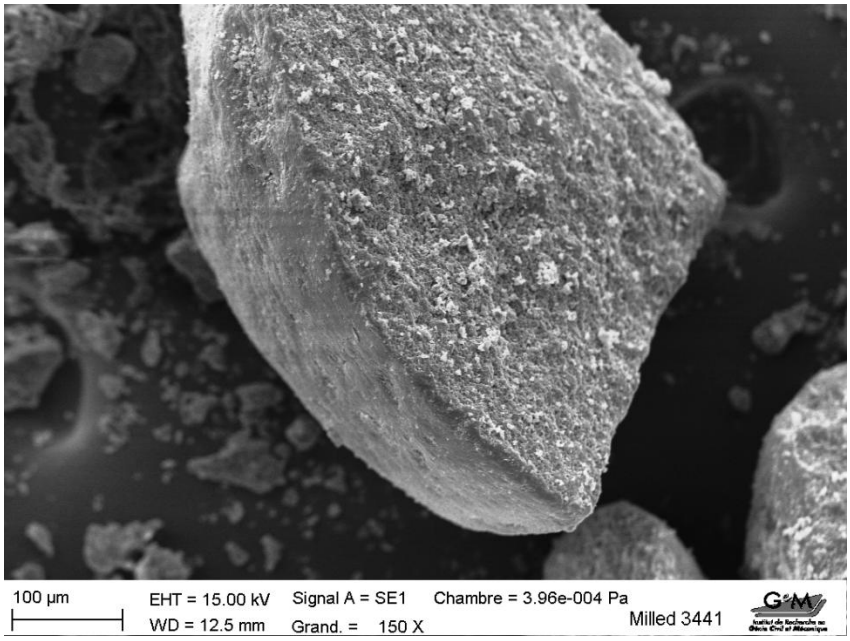


(b)

Figure 3. 7 SEM images of Actisaf STD granules (a) cross sectional view (b) section of yeast cell observed in the electronic microscope x10 000 obtained from Lesaffre Human Care and Feed Additives (France).



(a)



(b)

Figure 3. 8 SEM images of Milled 3441 dried yeast (a) x 60 magnification (b) x 150 magnification, obtained from Lesaffre Human Care and Feed Additives (France)

Actisaf STD granules exhibit a smooth surface (Figure 3.7a) and are spherical in shape, confirmed by the sphericity results. The composition of Actisaf STD (Figure 3.7b) includes; cellular residues which fill the voids between cell surfaces, the outer layer which has resisted the conditions from drying process and other cellular residues which are only made up of yeast cell walls. Large granules also have an area full of air inside (pores). Milled 3441 granules are smaller in size and more irregularly shaped than Actisaf STD (Figure 3.8a). As a result of the milling process, granules possess no internal pores and are of a wide range of sizes (3.8b).

3.3.3 Determining the mechanical properties of single primary particles using a micromanipulation technique

The forces required to compress single particles of MCC and DCP to rupture were measured using a micromanipulation technique. A schematic diagram of the equipment is shown in Figure 3.9.

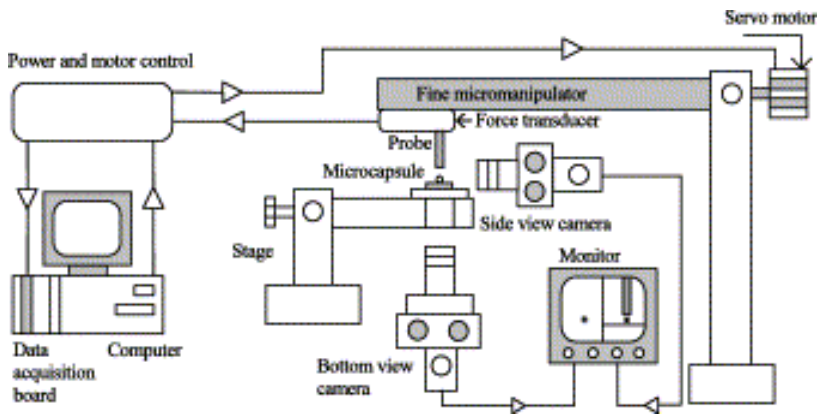


Figure 3. 9 Schematic diagram of the micromanipulation rig (Sun et al. 2002)

This technique is based on diametric compression, where a single particle is crushed between two parallel surfaces and the load required to cause fracture is measured. Before compression, the micro-particles were put on a slide and then placed on the stage and beneath the probe, which was made from a borosilicate glass rod with a flat end which made contact with the tested particle, as shown in Figure 3.10. The location of the stage could be positioned to run the experiment. The probe was connected to a force transducer, which was set to move vertically at a selected speed of 2 μm s^{-1} for all tests. The load applied on the single primary particles was measured by the force transducer (Models 400A, 402A and 405A, Aurora Scientific Inc.,Canada).

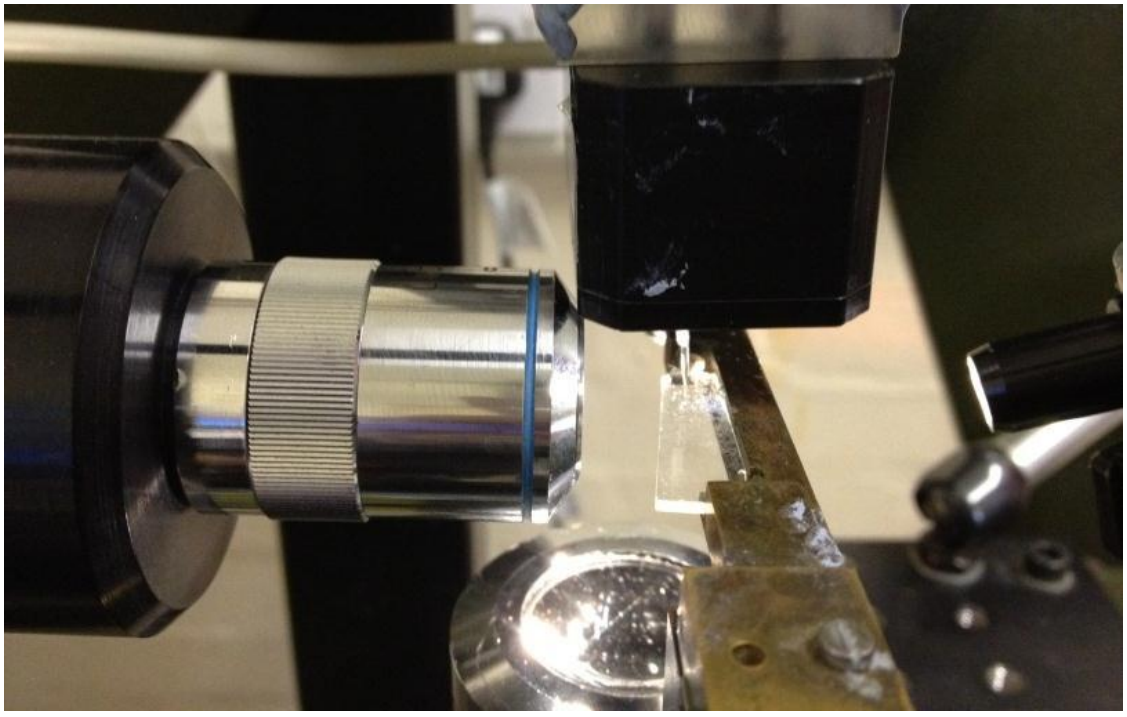


Figure 3. 10 Picture of the micromanipulation rig with the sample placed on the glass plate

The force transducer connected for the compression of DCP particles can exert a maximum force of 50 mN and that for compression of MCC particles, 500 mN. The compressive displacements were calibrated using the transducer and probe compliance. This was used to evaluate the deformation and the fracture behaviour of single particles under compression. Approximately 30-50 particles of each sample were measured to give statistically representative results and rupture values, expressed as mean ± 95% CI. In order to take into account the range of particle sizes, the rupture force, *F*, was used to calculate a nominal rupture stress:

$$\text{Nominal rupture stress} = \frac{4F}{\pi D^2} \qquad \textbf{Equation 4.3}$$

where *D* is particle diameter.

3.3.4 Determining the mechanical properties of single dried yeast granules using a Zwick/Roell Z030 mechanical tester

From preliminary studies, it was found that yeast granules and milled yeast particles would not rupture by micromanipulation. As a consequence, a similar approach to the micromanipulation technique was set up using the Zwick/Roell Z030 mechanical tester (Figure 3.11). A 100 N load cell with a 21.5 mm diameter was used to compress granules, using a speed of 0.01 mm s^{-1} The parameters for the compression tests, such as displacement and force were obtained by the programme testXpert® (Zwick/Roell, UK.). The nominal rupture stress was calculated according to Equation 3.3.

Figure 3. 11 Compression of single dried yeast granule using a Zwick/Roell Z030 mechanical tester

3.3.5 Formulation of single component tablets

Tablets consisting of single component excipients were produced in order to determine their compression behaviours. 0.5 g of each excipient (MCC, DCP, Milled 3441 and Actisaf STD) was individually weighed out and tablets were produced as described in *Section 3.2.3.3*. These were tested for their cell viability (in the case for Milled 3441 and Actisaf STD) and their mechanical strength as outlined in *Sections 3.2.2 and 3.2.4.2.*

3.3.6 Formulation of tablets consisting of binary mixtures

Tablets containing binary mixtures were produced according to Table 3.3 in order to determine the compaction behaviour of yeast granules with two typical pharmaceutical powder excipients as described in 3.3.5.

Table 3. 3 Various binary formulations comprising of Microcrystalline cellulose (MCC), Dibasic calcium phosphate (DCP) and Milled 3441 in different combinations to form 1 g tablets.

Formulation Number	Amount of Excipient %(w/w)		
	MCC	DCP	Milled 3441
1	50	50	-
2	50	-	50
3	-	50	50

3.3.7 Formulation of tablets consisting of tertiary mixtures

Tablets of combinations of tertiary mixtures consisting of powder excipients and yeast granules were produced according to Table 3.4.

Table 3. 4 Tertiary formulations comprising of (MCC), and Dried Yeast Milled 3441 to produce tablets.

Formulation Number	Amount of Excipient %(w/w)		
	MCC	DCP	Milled 3441
4	33.33	33.33	33.33

The tablets were produced and tested for their mechanical properties and cell viability. The tensile strength of these tablets and those consisting of binary and single components were compared with the mechanical properties of single particles. A hypothesis can be made regarding the mechanical properties of primary particles. The nominal rupture stress of primary particles can be related to the tensile strength of single component tablets.

3. 4 ANALYSIS OF TABLET COMPACTION DATA USING EMPIRICAL MODELS

3.4.1 Force versus displacement curves

Force verses displacement data were retrieved from the software attached to the Zwick/Roell mechanical tester. In order to take into account the different sizes of the tablets and variability in starting position (i.e. initial height), stress strain curves were produced as described below.

3.4.2 Stress versus nominal strain curves

The engineering nominal strain ε was calculated using Equation 2.2.

$$\boldsymbol{\varepsilon = \frac{h_i - h_p}{h_i}}$$

The stress was calculated using the following equation:

$$\boldsymbol{stress = \frac{F}{\pi r^2}}$$

.

Stress-strain curves were produced for the compaction of various formulations. These curves also formed the basis for the application of empirical models to the compression data as discussed below.

3.4.3 Kawakita model

The compaction behaviour in terms of pressure versus displacement of powder bed (volume reduction) was investigated. Stress versus nominal strain curves were fitted using the Kawakita model (Kawakita 1970) according to Equation 2.1:

$$\frac{\boldsymbol{\sigma}}{\boldsymbol{\varepsilon}} = \frac{\mathbf{1}}{\boldsymbol{ab}} + \frac{\boldsymbol{\sigma}}{\boldsymbol{a}}$$

3. 5 OPTIMISATION OF TABLET FORMULATION CONTAINING MILLED 3441

The amount %(w/w) and ratio of powder excipient included within a tablet formulation can have an effect on the final tablet properties and functionality such as tensile strength and probiotic health benefits. The influence of processing conditions such as compaction force and compaction speeds on tablet properties was also investigated as described below.

3.5.1 Varying the proportions of Microcrystalline Cellulose in the powder blend formulation

The proportion of MCC included in the formulation was varied whilst keeping a fixed amount of Milled 3441 yeast and Mg St. Tablets were produced as described in Section *3.2.3.3* according to Table 3.5. The resulting tablets were tested for their mechanical properties and cell viability.

Table 3. 5 Probiotic tablet formulations with varying proportions of Microcrystalline Cellulose (MCC) and Dibasic Calcium Phosphate (DCP) with a constant amount of Milled 3441 and Magnesium Stearate (Mg St).

Formulation Number	Amount of Excipient (%(w/w))			
	Milled 3441 yeast	MCC	DCP	Mg Stearate
5	50	0	49.25	0.75
6	50	12.31	36.94	0.75
7	50	25	24.25	0.75
8	50	36.94	12.31	0.75
9	50	49.25	0	0.75

3.5.2 Varying the yeast: powder blend ratio

Upon the selection of the powder blend composition, the formulation was further developed by varying the proportion of yeast. The effect of this inclusion on tablet properties (tensile strength and cell viability) was investigated. Tablets of different formulation compositions were prepared according to Table 3.6 and tested by methods outlined in *Sections 3.2.2 and 3.2.4.2.*

Table 3. 6 Tablet formulations comprising of various Milled 3441 yeast: powder bend ratios, final tablet weight 1 g. The powder blend composition is based on Formulation 7 (refer to Table 3.5)

Formulation Number	Composition (%(w/w))	
	Yeast	Powder Blend
10	0	100
7	50	50
11	60	40
12	75	25
13	85	15
14	100	0

3.5.3 Tablets produced at varying compaction pressure

The effect of tablet compaction pressure on tablet properties, such as tensile strength and cell viability was also investigated. A mixture of dried yeast granules and powder blend in the ratio of 75 %(w/w) and 25 %(w/w), Formulation 12 (Table 3.6), was produced and compressed into tablets. Tablets were compressed as outlined above over a range of compaction forces of 12 to 20 kN (pressures of ca. 95 to 150 MPa). For each test of tensile strength and cell viability, tablets were produced in triplicates with results expressed as mean ± 95% CI.

3.5.4 Speed- sensitivity test

The compaction speed might play a part in determining tablet properties, and thus is an important factor to consider during formulation optimisation. Powder particles react differently to compression at different speeds and it is important to determine these effects. Tablets were produced according to Formulation 12 (Table 3.6) using compression speeds from 1 to 60 mm min^{-1} and were tested for their tensile strength and cell viability.

3. 6 TABLET TESTING OF THE SELECTED PROBIOTIC FORMULATION (MG03)

Upon the selection of the final formulation for probiotic tablets, further testing of these tablets were conducted in order to ensure the product is fit for market and consumers. Further work involved conducting dissolution and friability tests according to the methods and specifications described in the Ph. Eur. In addition, for a product to reach the market, it is important to consider the storage stability over time and in various conditions. The methods for these tests are outlined below and the results are discussed in Chapter 6.

3.6.1 Dissolution test

Determining the dissolution rate of tablets provides significant information about the tablet regarding *in vitro* yeast release and for the prediction of the yeast release profile in the gastrointestinal tract (GI) tract, specifically the small intestine. This is critical as a sufficient number of viable cells need to be present and released at the required site of action, for the probiotic benefits to occur. The protocol followed was that given in the Ph. Eur. 5.0, Chapter 2, Section 2.9.3. To summarise, a USP dissolution apparatus was used where one tablet was placed in a basket rotating at a speed of 100 rpm, immersed in 500 mL medium maintained at 37°C (Figure 3.12).

Figure 3. 12 Dissolution apparatus using a basket method

The medium was prepared by dissolving 3 Phosphate Buffered Saline (PBS) tablets (Sigma-Aldrich, UK) in 600 mL deionized water to produce a pH of 6.8. 1 mL samples from each of the 6 media were taken at 10, 15, 20, 40, 60, 90, 120, and 150 min. Samples were diluted and plated as described above (Section *3.2.2*).

3.6.2 Friability

This test was intended to determine, under defined conditions, the friability of uncoated tablets, the phenomenon whereby tablet surfaces are damaged and/or show evidence of lamination or breakage when subjected to mechanical shock or attrition. This test was conducted according to the method outlined in Ph. Eur. (5.0) Section 2.9.7 using a friability machine at Aston University, UK (Sotax F2 USP Friability Tester, Switzerland). 10 tablets were accurately weighed and placed in the rotating drum. In order to take into account the

large size of tablets (approx. 13 mm in diameter), the drum was adjusted so that the angle formed a 10° angle to the horizontal as stated in the Pharmacopeia. The drum was rotated 100 times, tablets removed and accurately weighed again. The friability was expressed as the average loss of mass (% weight loss) and was calculated as a percentage of the initial mass. Tablets which had an average weight loss of less than 1% passed the friability test, as the product is robust enough to withstand handling and agitation caused during transportation.

3.6.3 Storage stability

Stability testing is of great importance as it provides evidence on how the quality of a product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light. The significance of this was as a result of the known negative effects external environments can have on the viability of probiotic microorganisms, resulting in a short shelf life (Thoorens et al. 2014). The data obtained are also used to establish a shelf life of the product and help form consumer guidelines for storage conditions. Storage stability tests were planned at Lesaffre in a long-term study consisting tablets (produced according to Formulation 7; 50% dried yeast, 25% MCC, 24.25% DCP and 0.75% w/w Mg St) placed in storage ovens of different temperatures (25, 30 and 40 °C) and relative humidity (60, 65 and 75 %RH, respectively). However this test was not carried out due to business reasons.

A small scale storage stability study was also conducted in-house (University of Birmingham). Tablets with the same composition were placed in plastic containers and stored in a 4°C fridge and Birmingham Science City Laboratory (University of Birmingham, UK) at

room temperature conditions (18°C and 40 +/- 10% RH). Tablets were removed at 2, 4 and 8 week time-points and tested for their cell viability as described above. Samples were taken in triplicate and results expressed as mean ± 95% CI.

3.6.4 Reproducibility quality test

In order to determine the consistency of tablet quality across a batch production, 20 tablets containing MG03 probiotic yeast were produced according to Formulation 7 (Table 3.5) using a powder blend produced comprising of the same excipients. The quality was determined by obtaining the yeast cell viability results post tabletting. The tablet numbers tested were 1, 5, 10, 15 and 20.

3. 7 CONCLUSION

This chapter describes the detailed methodologies used to investigate the formulation, production and testing of probiotic tablets containing dried yeast. All the yeast samples were screened and based on the tensile strength results (mean tensile strength of 1.3 ± 0.1 MPa); Milled 3441 sample was selected to take forward to develop a formulation and use to understand compression properties. In subsequent chapters, the results will be presented and discussed.

CHAPTER 4: PREDICTING THE COMPACTION BEHAVIOUR OF POWDER DURING TABLETTING FROM PRIMARY PARTICLE PROPERTIES

4.1 INTRODUCTION

Tablet strength is a vital parameter that determines the mechanical performance of the product from manufacturing to consumer handling (Kuentz 1999) and is commonly measured using diametrical compression (refer to Chapter 3, Section 3.2.4.2) (Fell & Newton, 1970). The mechanical strength of a tablet is dependent on the mechanical properties of individual primary particles and bonding (Yap et al. 2008). Therefore, it is vital to form an understanding of such particles and its mechanical strength.

During compaction, particles undergo a series of stages including the initial stage: rearrangement, where particles within the die cavity occupy the spaces between particles (Jivraj et al. 2000). The mechanism of rearrangement depends on the mechanical properties of primary particles. The second stage, deformation, is important and can influence the quality of the final tablet. At this point, due to the filling of all voids, no further rearrangement can take place and particles deform in various ways. Particles can exhibit deformation characteristics such as elastic, visco-elastic, or plastic and can include fracture or a combination of these mechanisms. Elastic behavior is temporary where particles temporarily take the shape whilst under load and return back to its original form upon the removal of the pressure load (Alderborn 2002). Alternatively, particles can undergo permanent deformation via plastic/ brittle fragmentation, where particles remain deformed in the shape whilst under pressure load. During fragmentation particles may break under

increasing pressure load into smaller fragments. The rupture of single particles during the compaction of a powder bed can play a part in the formation of a tablet during the tabletting process (Yap et al. 2008) since fragmented particles creates new surfaces available for bonding with other particles. It is essential that the particles deform plastically or rupture since stored elastic strains act to weaken a tablet on release (Roberts & Rowe, 1987a). As a result, the study of primary particles is necessary as a way to gather critical information to help understand the tablet compaction process of specific particles.

In literature, mechanical properties of particles can be characterised in many ways including: uniaxial confined compression of a powder, testing of compacts (e.g. indentation and bending tests) and testing of single particles e.g. by compression loading or nanoindentation (Nordström et al. 2012). A comprehensive procedure to characterise and provide a classification on the mechanical properties of tablets was suggested by Roberts et al. (1987), a procedure in which two of the approaches were combined i.e. powder compression and compact testing. A large range of pharmaceutical materials were experimentally tested by producing tablets whilst obtaining the lower and upper punch displacement/ time profiles. This raw data was then fitted to a theoretical model using Heckels plot as a predictive tool for compaction behaviour (Roberts et al. 1987).

Given the small size (20-90 µm) of many primary particles, a micromanipulation technique is necessary to determine the rupture stress and other mechanical properties. The technique has been used to characterise the mechanical properties of many single biological and non-biological particulate materials, including yeast (Mashmoushy et al., 1998), bacteria (Shiu et al., 1999) and pharmaceutical excipients (Yap et al., 2008). It is based on diametrical compression, similar to that used for tablets, in which a single particle is placed between two

parallel surfaces and the force required to cause rupture is measured. Larger particles (> 500 µm) can be characterised for mechanical strength using uniaxial compression of particles, which provides deformation data. This can be evaluated using Kawakita analysis (1970) to gain information regarding compression behavior. A relationship has been found between the nominal rupture stress of single particles and the theoretical strength derived from the Kawakita equation (Kawakita 1970, Adams et al. 1994, Yap et al, 2008), as shown by Figure 4.1.

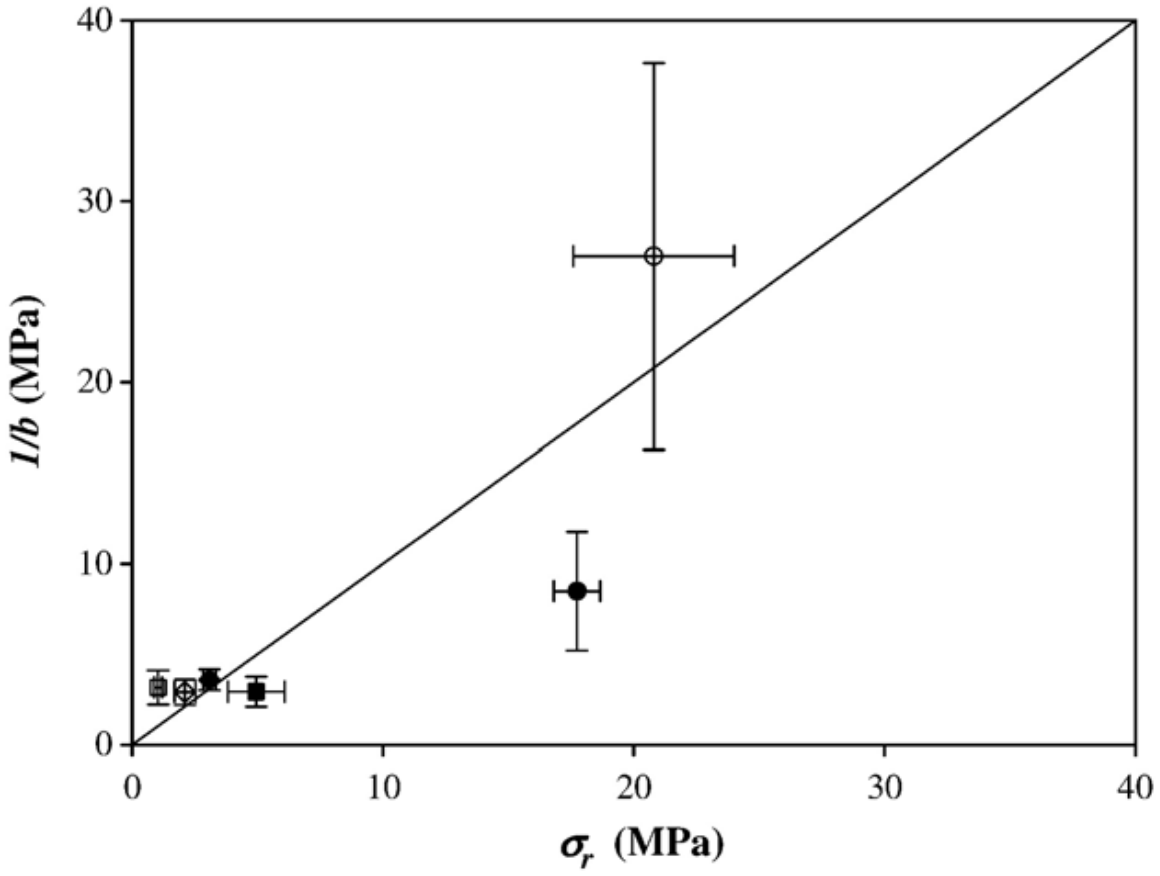


Figure 4. 1 Relationship between the Kawakita parameter, 1/b, and the mean nominal rupture stress of the particles, σr, for Eudragit® L100-55 (○),Eudragit® L100 (◇), Eudragit® S100 (□), Advantose™ 100 (●), calcium carbonate (♦) and Starlac™ (■). Extracted from Yap et al. 2008)

Based on the preliminary results presented in Chapter 3, Milled 3441 produced tablets of high tensile strength values and Actisaf STD granules showed high cell viability data of granules. Consequently, these samples were used for further work in this chapter. The objectives of the research described in this chapter, was to characterise particles with regards to their size and shape and then to relate the mechanical properties of primary particles of powder excipients and dry yeast granules (by using the micromanipulation technique) to their compaction behaviour.

4.2 RESULTS

4.2.1 Characterisation of primary particles

4.2.1.1 Particle size

Particle size and sphericity distributions were determined using image analysis (QICPIC) (refer to Chapter 3, Section 3.3.1.1). The particle size of powder excipients (MCC and DCP) were found to be significantly smaller (p<0.05) than dried yeast particles, with Actisaf STD granules being the largest (Figure 4.2).

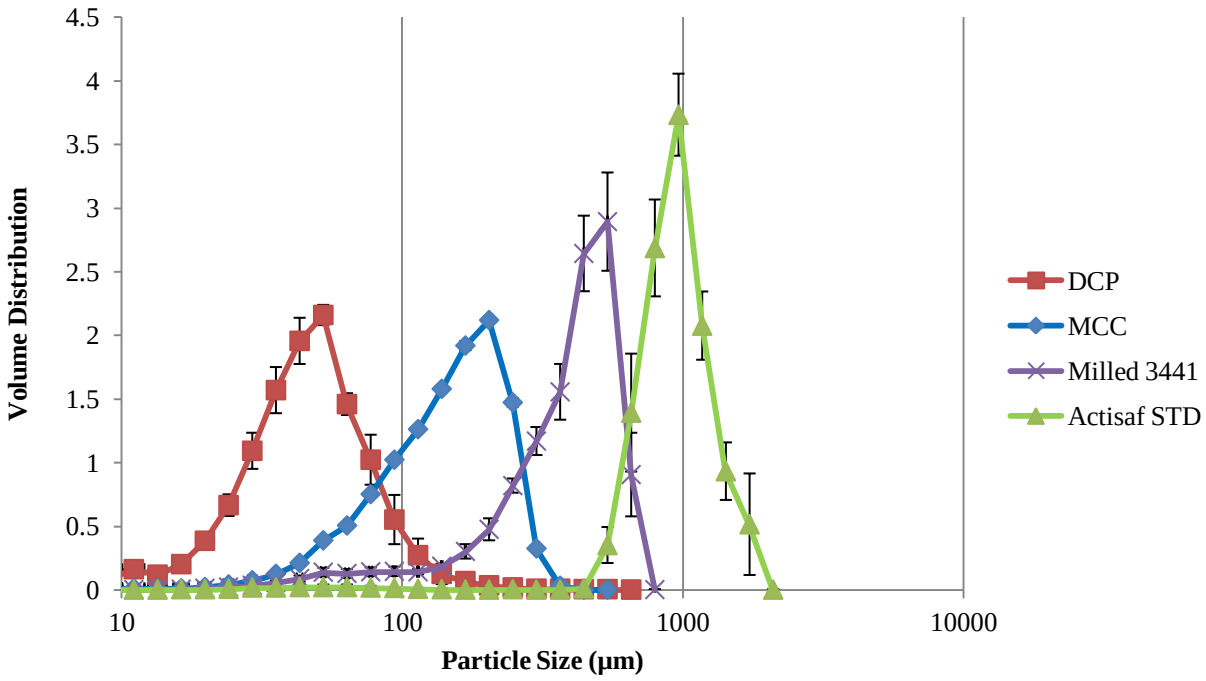


Figure 4. 2 Average particle size distribution curves for excipients and dried yeast particles measured using a QICPIC image analyser. Mean ± CI 95% (n=3).

The diameters of particles measured by an electronic digital calliper (Mitutoyo, U.K.), are recorded in Table 4.1.

Table 4. 1 Mean diameter of single powder particles (MCC and DCP), and dried yeast granules (Actisaf STD and Milled 3441). Mean ± 95% CI (n= 30, 37, 50, 50, respectively).

Excipient	Particle diameter (µm)
MCC	93 ± 10
DCP	22 ± 2
Actisaf STD	1252 ± 113
Milled 3441	749 ± 23

It was found that sphericity of particles for the excipients tested, varied across the particle size range. Excipients were sieved accordingly to ensure particles exhibited a sphericity ≥0.8 in order to obtain some consistency between the different materials being tested; MCC (Figure 4.3) sieved through a 212 µm mesh, DCP (Figure 4.4) through a 45 µm mesh and Actisaf STD granules (Figure 4.5) sieved through a 500 µm mesh. MCC showed a trend of increasing sphericity with decreasing particle size. DCP particles showed a similar pattern of increasing sphericity with decreasing particle size, however, at a particle size of 150 µm and above, particle are more spherical again. The whole particle size range of Actisaf granules exhibited a sphericity of 0.8 and above, with greatest sphericity seen at size 400 µm and larger. For Milled 3441 yeast granules, the whole particle size range exhibited sphericity ≥0.85. In addition, sieving was also conducted to obtain particles large enough for easy compression testing using the Zwick Instron.

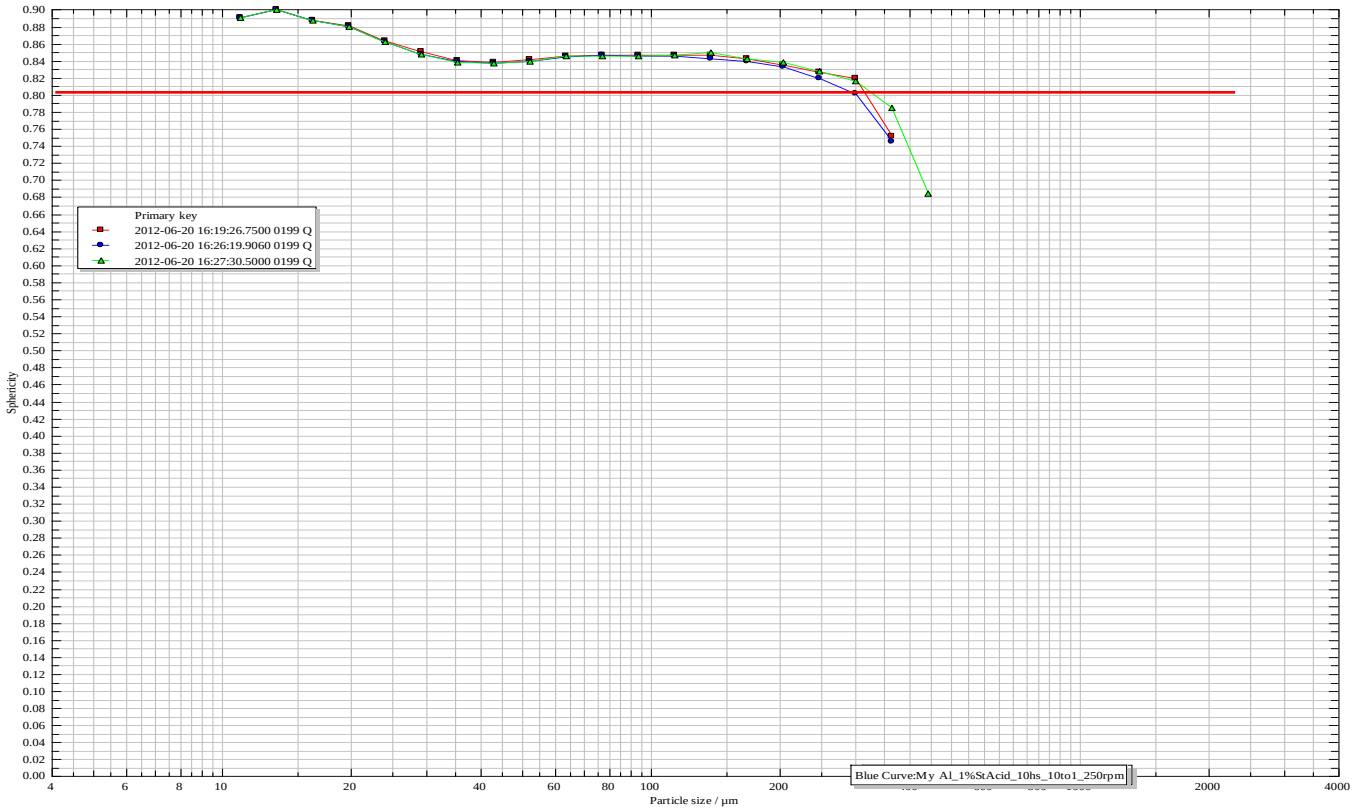


Figure 4. 4 Sphericitiy graph for Microcrystalline Cellulose particles obtained from a QICPIC Image Analyser (triplicate runs).

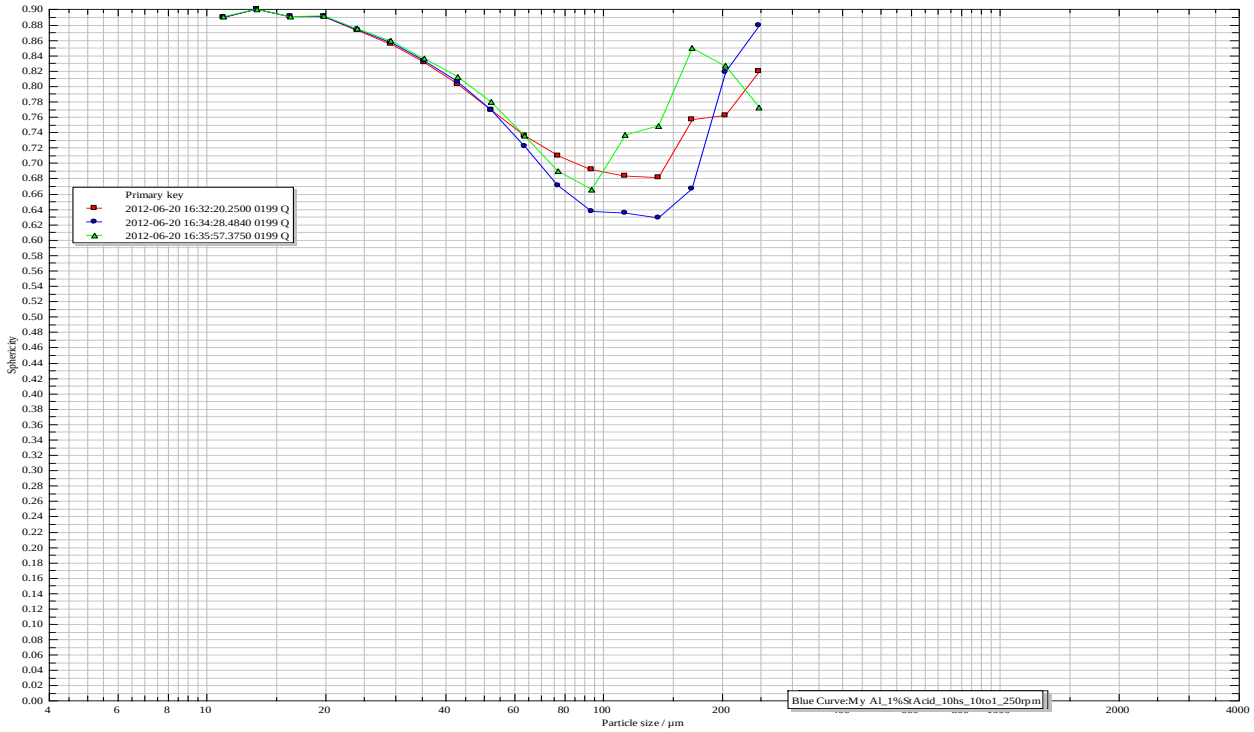


Figure 4. 3 Sphericity graph for Dibasic Calcium Phosphate (anhydrous) particles obtained from a QICPIC Image Analyser (triplicate runs).

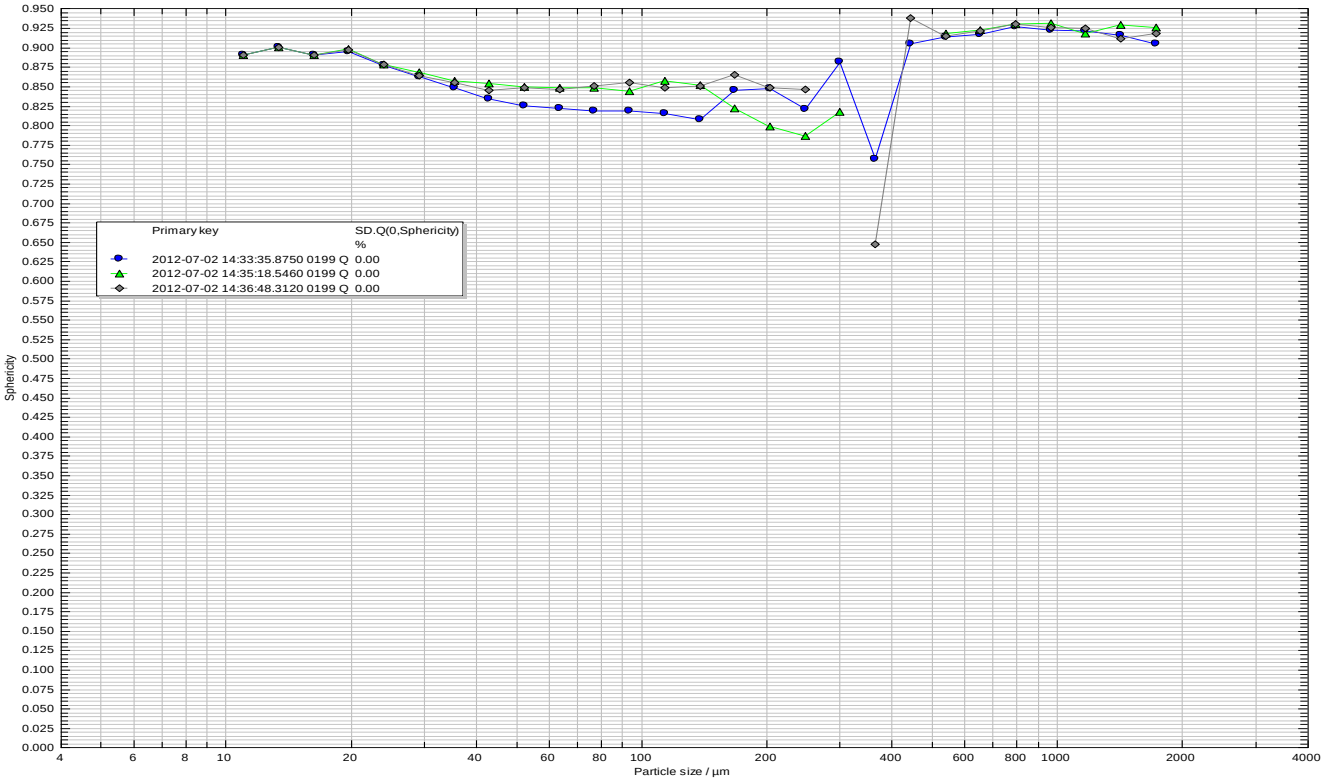


Figure 4. 5 Sphericity graph for Actisaf STD granules obtained from a QICPIC Image

4.2.2 Determining the mechanical properties of single primary particles

Individual primary particles of excipients and yeast granules were tested for their mechanical properties as outlined in Chapter 3 Section 3.3.

4.2.2.1 Mechanical strength of powder particles using micromanipulation technique

The typical force- displacement curve illustrating the compression behaviour of a single DCP particle is shown in Figure 4.6. Initially, as the force applied increases (A to B), the particle undergoes initial compression until a limit is reached. At point B the particle ruptures. As the probe continues to move further, compression of the broken particle (debris) occurs as seen in curve C-D. The curve confirms the reported brittle behaviour of DCP particles under compression (Jivraj 2000, Roberts 1987).

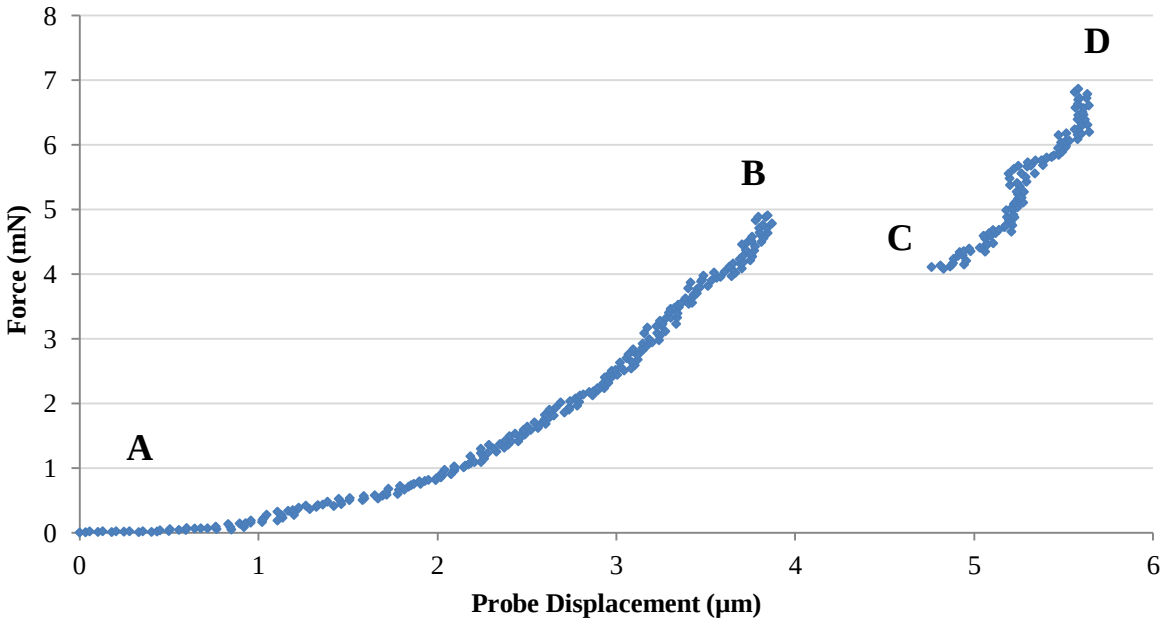


Figure 4. 6 Typical force-displacement curve for the compression of a single Dibasic Calcium Phosphate particle using a micromanipulation technique (initial diameter = 23.2 µm) using compression speed of 2 µm s-1. A to B shows the initial compression of the particle followed by particle rupture at point B. Compression of the broken particle (debris) continued as seen in curve C-D.

The compression of single MCC particles showed different properties than those seen with DCP particles (Figure 4.7). MCC particles did not rupture under compression which was not surprising given its reported plastic deformation behaviour (Jivraj 2000, Roberts 1987, Westermarcka et al. 1999).

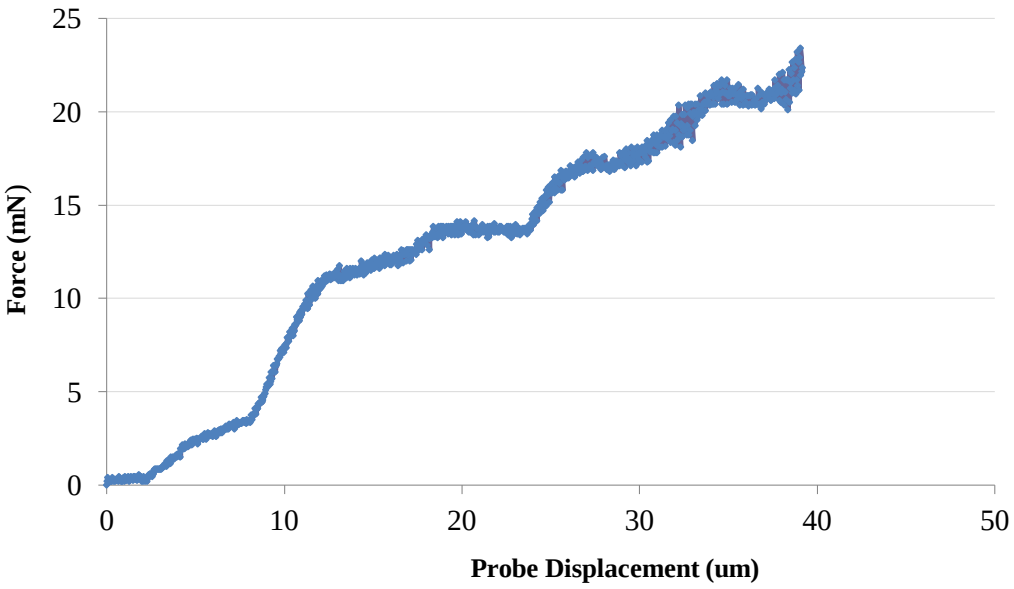


Figure 4. 7 Typical force-displacement curve for the compression of a single Microcrystalline Cellulose particle using a micromanipulation technique (initial diameter = 108 µm) at a compression speed of 2 µm s^{-1}.

4.2.2.2 Mechanical strength of dried yeast granules using a Zwick Instron

The compression behaviour of dried yeast granule samples was determined by compressing a single granule between two plates until fracture (Chapter 3, Section 3.3.4). Like DCP, Actisaf STD and Milled 3441 (Figure 4.8) also ruptured under compression. By looking at the curvature, yeast granules behave in a similar way to DCP when under load; initial compression up to the point of fracture and compression of broken granules (debris) took place with increasing application of load. For Actisaf STD granules, the probe reached the granule causing it to fracture (shatter), after which the probe may continue to touch the residue (debris) hence the increase in force again. This suggests that Actisaf STD and Milled 3441 granules may exhibit brittle fracture behaviour during the process of tablet production. The slope of the force vs. displacement graph provides an indication of the hardness of the particle, using the linear region of the loading curve (Yap et al. 2008). This is a contributing factor during the tablet compression process and is considered for further work (discussed in Chapter 7, Section 7.2).

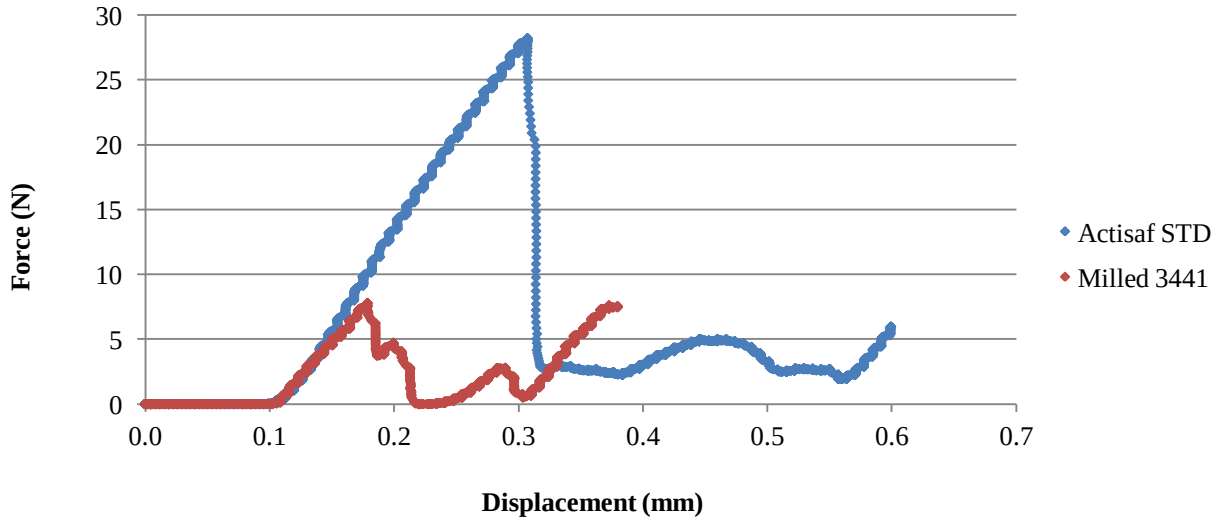


Figure 4. 8 Typical force-displacement curves for the compression of Actisaf STD (diameter = 1.46 mm) and Milled 3441 granules (diameter = 0.74 mm) using a Zwick Instron at a compression speed of 0.01 mm s^{-1}.

A summary of the mean rupture force and mean nominal rupture stress for single DCP particles, Actisaf STD and Milled 3441 granules is presented in Table 4.2. Once the size of the primary particles had been taken into account (by calculating the stress, defined in Chapter 3, equation 3.3), no difference ($p>0.05$) was seen between the mean nominal rupture stress of DCP particles, Milled 3441 and Actisaf STD yeast granules, indicating all materials exhibit similar mechanical strength. Investigations into the effect of the mechanical properties of primary particles on their compaction behaviour, is described below.

Table 4. 2 The mean rupture force and calculated nominal stress of single DCP particles (determined using the micromanipulation technique) and Actisaf STD and Milled 3441 granules using a Zwick Instron. Mean ± 95% CI (n= 37, 50, 50, respectively)

Material	Mean Rupture Force (mN)	Particle Diameter (µm)	Mean Nominal Rupture Stress (MPa)
DCP	11 ± 4	22 ± 2	24 ± 6
Milled 3441	7600 ± 1600	749 ± 23	17 ± 2
Actisaf STD	16000 ± 2000	1252 ± 113	15 ± 2

4.2.3 Production of single component tablets

Tablets of individual excipients were produced in order to determine their compaction properties according to methods outlined in Chapter 3, Section 3.3.5.

4.2.3.1 Determining the tensile strength of single component tablets

The tensile strength of tablets comprised of single excipients was used as an indicator of their mechanical strength properties. Tablets of pure MCC were strongest with mean tensile strengths (5.5 ± 0.1 MPa) significantly higher (*$p<0.05$)* than tablets of other excipients (Figure 4.9).

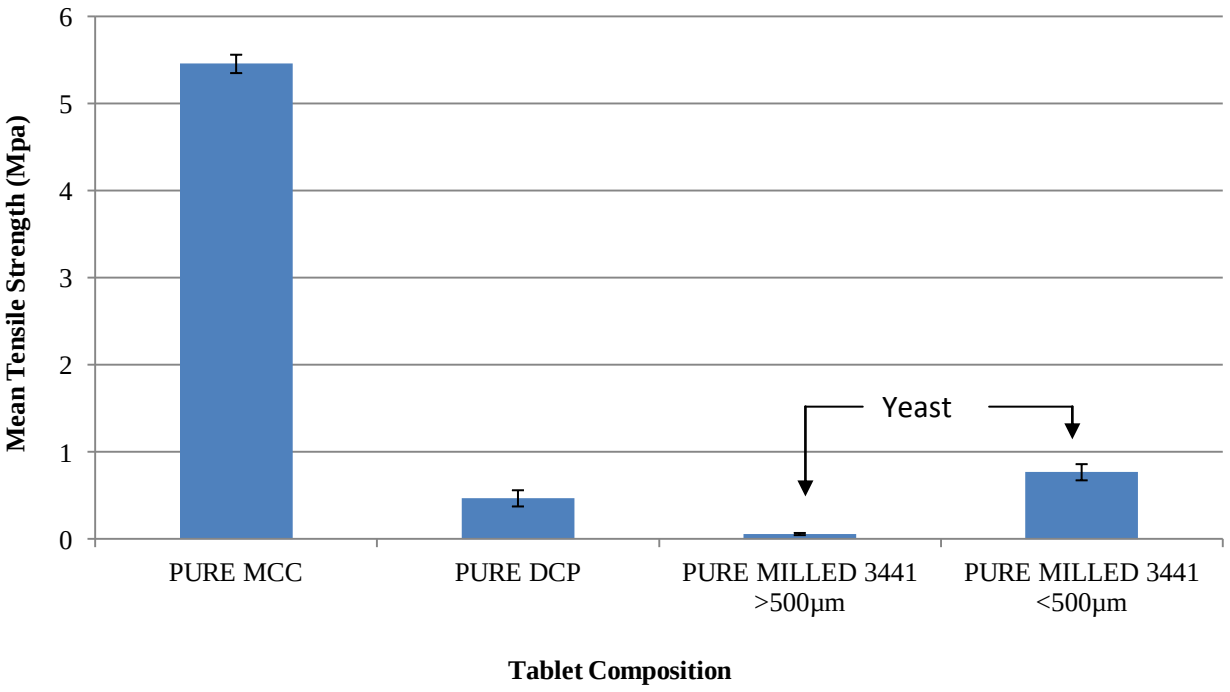


Figure 4. 9 Tensile strength (MPa) of tablets consisting of single components of MCC, DCP and Milled 3441 yeast produced and tested according to Chapter 3, Section 3.3.5. Mean ± 95% CI (n=10) using a compaction force of 14 kN (compression pressure of ca. 105 MPa) and speed of 10 mm min^{-1}.

This illustrates and confirms the excellent binding properties exhibited by MCC as reported by many authors, including Jivraj et al. (2000), Klevan et al. (2010) and Roberts et al. (1987). As seen with other excipients illustrating plastic deformation, the formation of permanent particle–particle contact regions is facilitated during compaction (Jain 1999).

Tablets of DCP alone exhibited lower mean tensile strengths, indicating poorer compaction properties as a result of the predominant brittle fracture deformation mechanism of the particles (Klevan et al. 2010, Rowe et al. 2009). Compacts of Actisaf STD did not form a rigid tablet and could not withstand handling indicating its poor binding properties. Therefore, Actisaf STD was not included in further studies in this chapter.

A significant difference ($p<0.05$) was observed between Milled 3441 yeast tablets of two granular size ranges i.e. < 500 µm and > 500 µm. Granules < 500µm produced tablets with higher tensile strengths: 0.77 ± 0.09 MPa and 0.06 ± 0.01 MPa, respectively. A possible explanation is that smaller granules provided a larger surface area for bonding and granule-granule interaction, resulting in stronger compacts (Jain 1999).

4.2.3.2 Determining the cell viability of single component tablets

The compaction of Milled 3441 (particle > 500 µm) did not form rigid tablets that could withstand handling, hence the cell viability could not be determined. Measurements of the cell viability of Milled 3441 yeast tablets (particle size < 500 µm) showed 1 log loss (5 x 10^8 ± 1 x 10^8 CFU/ g tablet) on compaction. It has been reported by Plumpton et al. (1986) that the functionality of yeast is significantly reduced during tabletting due to the pressures and this is consistent with the findings here. When studying the mechanistic effect on the inactivation of *S. cerevisiae* by high hydrostatic pressure, Brul et al. (2000) found data to suggest that intracellular membrane damage is the most likely initial target. This could provide a possible explanation for the cell viability loss of yeast granules during tablet compaction. When considering the formulation of tablets containing dried yeast granules, the cell viability post tabletting is an important factor to be considered, in order for the final product to exert its functionality. Therefore, the inclusion of powder excipients as a route to improve cell viability was investigated.

4.2.4 Tablets comprising of binary mixtures

Binary mixtures have been widely studied in order to determine and understand the compaction property of pharmaceutical excipients (Busignies et al. 2006, Frenning et al. 2009, Koynov et al. 2013). A similar approach was employed here with Milled 3441 yeast granules and two commonly used pharmaceutical excipients; MCC and DCP (Chapter 3, Section 3.3.6).

4.2.4.1 Determining the tensile strength of binary mixtures

The tensile strength of binary mixtures comprising of MCC and DCP, was found to be highest (2.85 ± 0.04 MPa) compared to mixtures of Milled 3441 yeast with MCC or DCP (2.00 ± 0.08 MPa and 0.68 ± 0.03 MPa, respectively), as can be seen in Figure 4.10. These results further highlight the excellent binding properties of MCC compared to DCP. As found by Jivraj (2000), DCP is best combined with MCC or starch, as also found here where the tensile strength increased from tablets of DCP alone (0.47 ± 0.09 MPa) to MCC-DCP binary mixture tablets (2.85 ± 0.04 MPa). This could also explain why stronger tablets are produced with a mixture of particles undergoing a combination of plastic and brittle deformation under load. This seems to be the case for MCC-Milled 3441 yeast tablets compared to DCP-Milled 3441 yeast, where all particles undergo the same mechanism of fragmentation due to the brittle nature of primary particles.

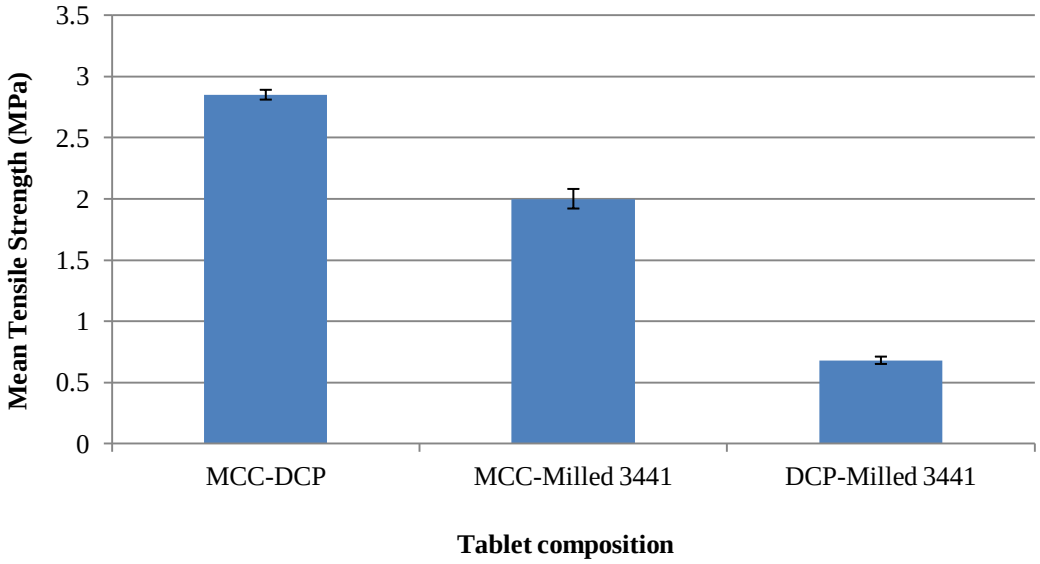


Figure 4. 10 Tensile strength (MPa) of tablets consisting of single components according to Table 3.3 (Chapter 3, Section 3.3.6) Mean ± 95% CI (n=10) using a compaction force of 14 kN (compression pressure of ca. 105 MPa) and speed of 10 mm min^{-1}.

4.2.4.2 Determining the cell viability of binary mixtures

The cell viability of yeast cells post tabletting was determined for tablets comprising of yeast granules and powder excipients in binary mixtures. Tablets of DCP- Milled 3441 yeast resulted in a higher cell viability ($1.2 \times 10^8 \pm 0.3 \times 10^8$ CFU /g tablet) than tablets of MCC-Milled 3441 yeast ($8.5 \times 10^7 \pm 1.5 \times 10^7$ CFU /g tablet). A possible explanation for this may be the packing behaviour of MCC particles which undergo greater deformation (Figure 4.12), resulting in greater damage to yeast granules than those interacting with DCP granules which fragment (and deform less due to its brittle nature). It was found that the type of consolidation during compaction (plastic flow or brittle fracture) influenced survival of bacteria, plastic flow causing the greatest kill at low compression forces which could be the

case here too (Blair 1991). This is discussed further in Section 4.2.6 where the extent of deformation under load for MCC and DCP particles is analysed.

4.2.5 Tablets containing tertiary mixtures: effect on tensile strength and cell viability

Tablets comprising of a mixture of the three components; MCC, DCP and Milled 3441, were produced and analysed regarding their tensile strength and cell viability according to Table 3.4 (Chapter 3, Section 3.3.4). The tensile strength was 1.82 ± 0.09 MPa with cell viability of $2 \times 10^7 \pm 1 \times 10^7$ CFU /g tablet. With regards to the tensile strength, these results confirm further the benefits of the inclusion of MCC in a tablet formulation containing DCP, as the strength was found to increase by 63% (from binary DCP-Milled 3441 yeast tablets to MCC-DCP-Milled 3441 yeast tertiary tablets). However, a formulation comprising of all three components had a detrimental effect on the cell viability. This could be due to yeast granules being damaged to a further degree, as a result of the different fragmentation behaviour of MCC particles (plastically) and DCP (brittle fracture) occurring during tablet compaction.

4.2.6 Compaction analysis and prediction of compaction behaviour

The compaction of single particles and bulk mixtures of powder excipients and yeast granules was analysed to determine if a correlation exists between the mechanical properties of primary particles and their resulting compaction behaviour (as described in Chapter 3, Sections 3.3.3- 3.4.3).

4.2.6.1 Force versus displacement curves

Tablets comprising of single, binary and tertiary compositions were produced, from which resulting force displacement curves were obtained, shown in Figure 4.11. The data show that MCC particles were displaced to a greater degree (6.9 ± 0.1 mm displacement) compared to all other mixtures highlighting the plastic deformation, which presumably led to its excellent binding properties. These data were used to produce stress-strain curves (refer to Chapter 3, Section 3.4.2 for methods) and plotted according to the Kawakita model to understand the compaction behaviour of particles further.

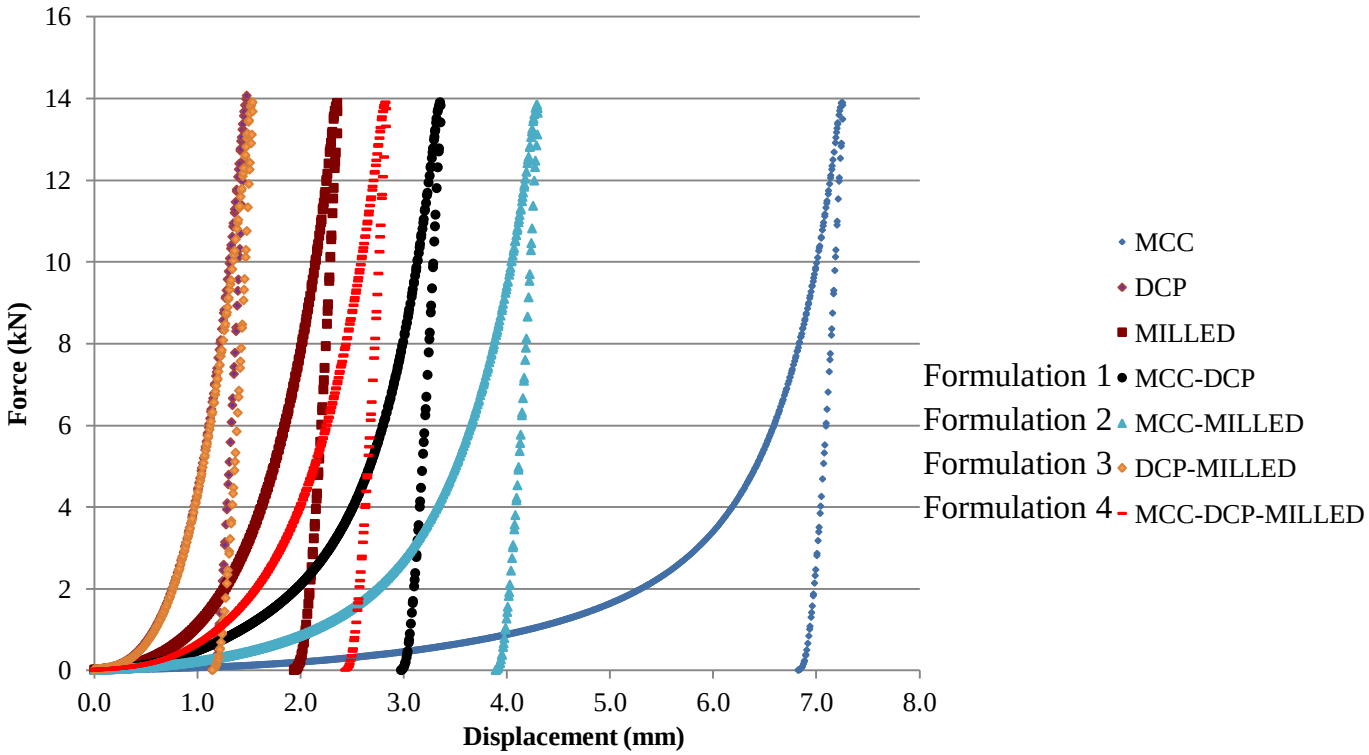


Figure 4. 11 Typical force displacement curves for the compaction of tablet compositions comprising of single, binary and tertiary mixtures produced using a compaction force of 14 kN (ca. 105 MPa pressure) according to the method in Chapter 3, Sections 3.3.5- 3.3.7.

4.2.6.2. Fitting to the Kawakita model

The Kawakita model predicts that there is a linear correlation between σ/ ε and σ:

$$\frac{\sigma}{\varepsilon} = \frac{1}{ab} + \frac{\sigma}{a}$$

Figures 4.12 and 4.13 show typical plots for applying this correlation in this linear form, for MCC as a single component tablet and in a binary mixture with Milled 3441 yeast. A good agreement between the experimental data and the model can be seen and this was the case for all tablet compositions.

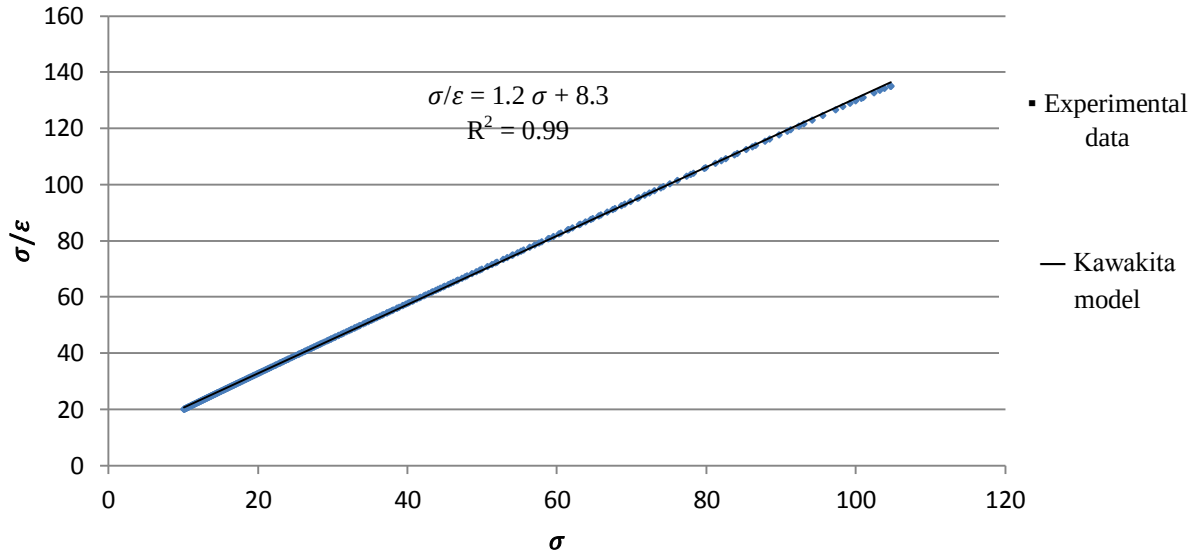


Figure 4. 12 Typical Kawakita plot for the compaction of MCC particles to 0.5 g tablets produced at a compaction force of 14 kN (pressure ca. 105 MPa) and compression speed of 30 mm min^{-1}.

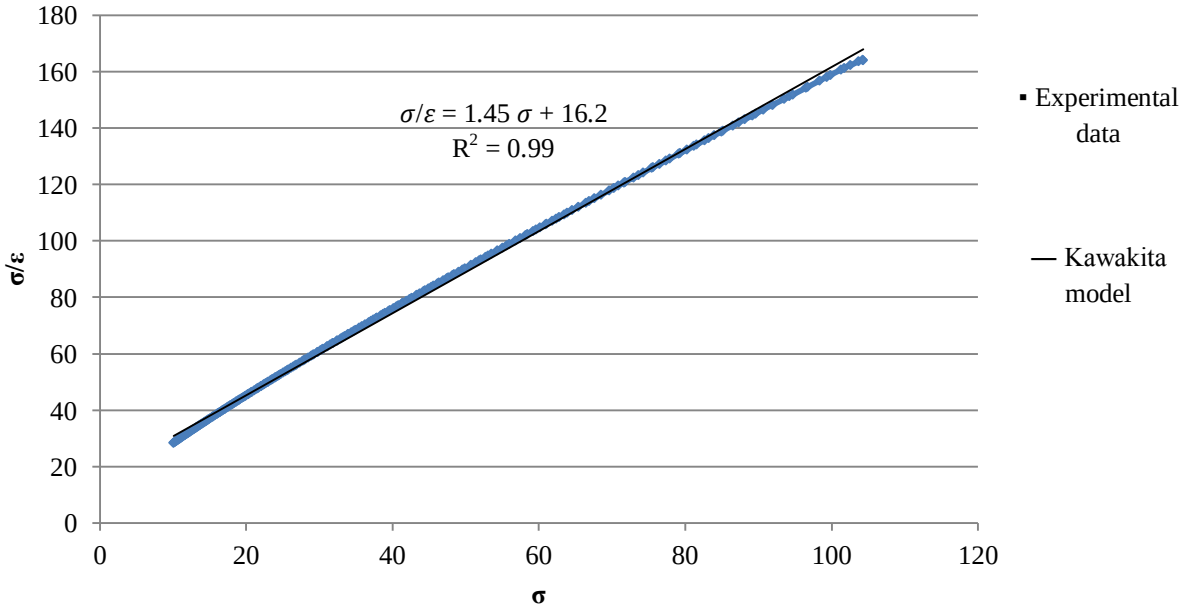


Figure 4. 13 Typical Kawakita plot for 0.5 g tablets comprising of 50 %(w/w) MCC and 50 %(w/w) Milled 3441 yeast as a binary mixture using a compression force of 14 kN (pressure ca. 105 MPa) and a compression speed of 30 mm min^{-1}

The Kawakita parameters were determined for the compaction of tablet compositions comprising of single, binary and tertiary mixtures (Table 4.3). Constant *a* was calculated by:

$$Kawakita\ constant\ a = \frac{1}{gradient}$$

and was used to calculate constant *b* by:

$$Kawakita\ constant\ b = \frac{1}{(intercept\ \times constant\ value\ a)}$$

Kawakita plots fit the data very well and look as expected, given the linear regression of data to the model (Nordström et al. 2012; Yap et al. 2008). Oyi et al. (2009) also investigated compaction properties of MCC and found a linear relationship with a correlation coefficient (R^2= 0.999) with the Kawakita model. This was also seen in work by Mazel et al. (2011), who found a good agreement between experimental and predicted data ("in-die" data, R^2 = 1.0 and "out-of-die" data, R^2 = 0.9997), proving that the Kawakita model was efficient to predict the volume reduction of MCC.

The value of constant *a*, is related to the initial bed voidage (Adams et al. 1994, Yap et al. 2008) and was found to be highest for MCC single component tablets and lowest for binary DCP-Milled 3441 yeast mixtures. In previous work, the Kawakita compression parameters were studied and it was found that powders expressing significant particle rearrangement, at low compression pressures, showed low values of parameter b^{-1} and high values of parameter *a* (Klevan et al. 2010). This seems to be the case for tablets of MCC where the greatest deformation produced tablets of high tensile strengths. On the contrary, the low value of parameter *a* obtained for tablets of DCP-Milled 3441 yeast suggests little particle rearrangement occurring, as discussed in Section 4.2.6.3. This is also highlighted by

the high 1/*b* values, which are associated with lower values of the degree of volume reduction (Frenning 2009). However, these relationships are empirical and the observations cannot be fully explained at this stage. The bonding and adhesion of particles during tabletting, as well as looking at particle surface properties would help form a better understanding of how particles behave during compaction. This presents a scope for future work to form further understanding of the relationships between Kawakita parameters *a* and *b* and particle compaction properties.

Table 4. 3 The extrapolated Kawakita parameters for various tablet compositions produced at a compression force of 14 kN (pressure ca. = 105 MPa). (Mean + 95% CI n=10) and Mean nominal rupture stress of DCP and Milled 3441 particles (Mean ± 95% CI n= 37 for DCP and 50 for Milled 3441).

Tablet Component	Kawakita Parameter		R^2	Mean Nominal Rupture Stress (MPa)
	a	1/b (MPa)		
MCC	0.816 ± 0.001	7.1 ± 0.2	0.999	-
DCP	0.61 ± 0.01	19 ± 1	0.994	24 ± 6
Milled 3441	0.58 ± 0.01	19 ± 1	0.996	17 ± 2
MCC-DCP	0.710 ± 0.001	14.174 ± 0.001	0.999	-
MCC- Milled 3441	0.689 ± 0.002	11.137 ± 0.002	0.998	-
DCP- Milled 3441	0.53 ± 0.01	26.875 ± 0.003	0.995	-
MCC-DCP-Milled	0.68 ± 0.02	17.430 ± 0.001	0.999	-

4.2.6.3 Prediction of compaction properties from primary properties

Since single MCC particles did not rupture and tablets of Actisaf STD did not form (refer to Sections 4.2.2.1 and 4.2.3.1, respectively), comparisons were made with Milled 3441 yeast granules and DCP particles only. No difference was seen between the experimental mean nominal stress values of single particles and the *1/b* Kawakita parameter values. Due to the lack of data points, the relationship between *1/b* and the nominal rupture stress of single particles could not be plotted and no strong conclusions could be drawn. As shown by the high error value, a significant variation in the nominal rupture stress of DCP particles was present. This is a commonly reported issue which may be due to the presence of defects, such

as cracks that are formed during the manufacturing of excipients (Yap et al. 2008). Yap et al. (2008) also investigated compression behaviour of various pharmaceutical excipients, using a micromanipulation technique and found a reasonable linear correlation between extrapolated *1/b* values plotted as the mean nominal rupture stresses of the corresponding single particles, with a gradient of 0.95 ± 0.30.

The relationship between the tensile strength of single component tablets and corresponding *1/b* values (indicating stress of primary particles) was also investigated (Table 4.4). A trend can be seen where a high *1/b* value results in tablets with low tensile strength. A hypothesis can be made where strong mechanical properties of primary particles do not break easily during compaction, thus do not deform or rupture easily, resulting in fewer new surfaces available for bonding. Consequently, the tensile strength of resulting tablets would be low. This is supported by Bashaiwoldu et al. (2011), who also found the failure stress of individual granules (Kawakita *b*) was inversely proportional to tensile strength. In addition, an attempt was made to correlate the tensile strength of single component tablets against the theoretical initial porosity (represented by Kawakita parameter *a*). In both cases, graphs were plotted (not shown here), but as seen earlier, no concrete conclusions could be drawn due to a lack of data points, which presents an opportunity for future work.

Table 4. 4 The correlation between the Kawakita 1/*b* value, Kawakita *a* value and the tensile strength of single component tablets produced at compaction force of 14 kN (pressure ca. = 105 MPa) and speed of 30 mm min-1. Mean + 95% CI (n = 10). (■ DCP, ● Milled 3441, ♦ MCC).

Excipient	**Tensile Strength (MPa)**	**Kawakita Parameter 1/*b* (MPa)**	**Kawakita Parameter *a***
MCC	5.46 ± 0.11	7.1 ± 0.2	0.816 ± 0.001
DCP	0.47 ± 0.10	18.9 ± 1.2	0.61 ± 0.001
Milled 3441	0.77 ± 0.90	18.6 ± 1.4	0.58 ± 0.01

4.3 CONCLUSION

A significant difference was found in particle size between MCC and DCP powder excipients and dried yeast granules, with Actisaf STD possessing the largest size and DCP particles the smallest. The impact of plasticity and brittleness of primary particles on the tensile strength of tablets was highlighted. The rupture force was determined for primary particles and found to be the highest for Actisaf STD granules. Due to the plastic behaviour of MCC during tabletting, particles did not show rupture under compression using the micromanipulation technique. The reported good binding properties of MCC was confirmed when tablets of single components were produced. MCC tablets resulted in tensile strength of 5.5 ± 0.1 MPa, which was significantly higher ($p<0.05$) than tablets of other components and further confirmed when binary mixtures were investigated. The final combination comprising of a tertiary mixture, exhibited results of a high tensile strength (1.82 ± 0.09 MPa), which was considered favourable due to the inclusion of DCP, a source of calcium in nutritional supplements (Rowe et al. 2009).

Upon fitting the compression data to the Kawakita model, the equation can be used to represent the compression data reasonably well, with a regression coefficient. To determine a correlation between primary particles and its corresponding Kawakita parameter 1/*b*, a wider range of compressible excipients would need to be tested. In addition, the overall conclusions that can be drawn are limited, as only the mean rupture stress data was available. The tensile strength is known to be related to the surface and bonding properties of particles (Eriksson and Alderborne 1995), but this was not investigated in this project, presenting a limitation. Nevertheless, an attempt to form correlations was still made and identifies a gap to bring

forward in future work. In addition, the compression data obtained of single particles could be analysed further, modelling the data to determine the elasticity properties of particles using the Hertz equation and determining the hardness of particles (Yap et al. 2008).

CHAPTER 5: FORMULATION OF PROBIOTIC TABLETS CONTAINING MILLED 3441- EFFECTS ON TENSILE STRENGTH AND CELL VIABILITY

5.1 INTRODUCTION

The mechanical properties of tablets are vital as they determine the performance of the final product, during manufacture and through to consumer handling. Tensile strength is considered to be the most important mechanical property and is measured for quality assurance during pharmaceutical production (Kuentz et al. 1999). As a result of its importance, the compaction of pharmaceutical powders has been widely researched, in order to gain an understanding of particle behaviour during direct compression (Kawakita 1971, Roberts et al. 1987, Yap et al. 2008).

The relationship between the packing or compression of powders and their fundamental physical properties is an important consideration in the field of powder technology. Tablet compression profiles and crushing-strength data provide useful information for limiting compression forces during tabletting and can provide information about lamination or capping (Jain 1999). As addressed in Chapter 4, Section 4.2.6, the compaction behaviour of powders has been widely studied in order to understand the mechanical properties of primary particles, to determine factors such as tablet strength. The successful production of tablets exhibiting good mechanical properties, depends upon the choice of excipients i.e. binder and filler and their proportions. The role of each excipient, as well as its mechanism of compaction under pressure, influences the mechanical properties of the final tablet (Jivraj et al. 2000, Yap et al. 2008). Therefore, it is important to achieve the right balance of excipients

undergoing brittle fracture (such as Dibasic Calcium Phosphate) and plastic behaviour (such as Microcrystalline Cellulose) by incorporating the right quantities of each (Jivraj et al. 2000, Roberts et al. 1987).

Compaction speed can play a part in determining final tablet properties, thus is an important factor to consider during formulation optimisation of tablets. Primary particles react differently to compression at different speeds, hence it is important to determine these effects. Roberts et al. (1987) found that when testing many inorganic material, including dicalcium phosphate dihydrate, all samples exhibited high yield pressures independent of strain rate, which is a characteristic of brittle fracture, their known compaction behaviour. On the other hand, the polymers tested (including Microcrystalline Cellulose) displayed low yield pressures with high strain rate sensitivities and compaction by plastic deformation (Roberts et al. 1987).

This chapter describes work investigating different formulations of probiotic tablets with regards to yeast and powder composition. Based on the preliminary results obtained from Chapter 3 (Section 3.2.4.1, Figure 3.3), Milled 3441 was selected for the work presented in this chapter, due to its good compaction properties (mean tensile strength of 1.3 ± 0.1 MPa). Preliminary studies suggested that a compaction force of 14 kN (pressure ca. = 105 MPa) and speed of 30 mm min^{-1} would be good choices and these remained fixed during the stages of formulation development. The choice of compaction force was also confirmed by Chan et al. (2002), who investigated the effects of compression force on the cell viability of probiotic bacteria and found that when the compression pressure was increased from 30 to 90 MPa, there was a gradual increase in loss of cell viability due to damage to cell walls. It was also concluded that when the compression pressure exceeded 90 MPa, damage not only occurred

to the wall of cells, but also to their membrane. However, yeasts and bacteria probiotic may not be expected to behave in the same way during tabletting. Considering this, a compaction force of 14 kN (pressure ca. 105 MPa) was selected, based on the tablet strength data obtained from the work conducted thus far. In addition to this, a compression speed of 30 mm min^{-1} remained fixed and was used during the formulation development stages.

Initially, an optimum powder blend composition was identified and was then used for further trials of tablets containing yeast granules, added in various proportions. The bulk compaction data of tablets containing varying ratios of yeast to powder blend were analysed using stress-strain curves, to calculate the parameters of the Kawakita model (refer to Chapter 3, Section 3.4). Upon the selection of a formulation, the processing conditions such as the compaction force and speed were then investigated for the production of probiotic tablets containing dried yeast granules (methods outlined in Chapter 3, Section 3.5.3 and 3.5.4).

5.2 RESULTS

5.2.1 Varying the proportions of Microcrystalline Cellulose in the formulation

5.2.1.1 The effect on tensile strength

Preformulation studies showed that for a compaction force of 6-25 kN (pressures ca. 45-187 MPa), dried Milled 3441 yeast granules alone did not compact into rigid tablets and did not meet the strength specification (except at unreasonably high forces of those above 26 kN), since the resulting tablets were unable to withstand handling as implied by low tensile strength values (Figure 5.1).

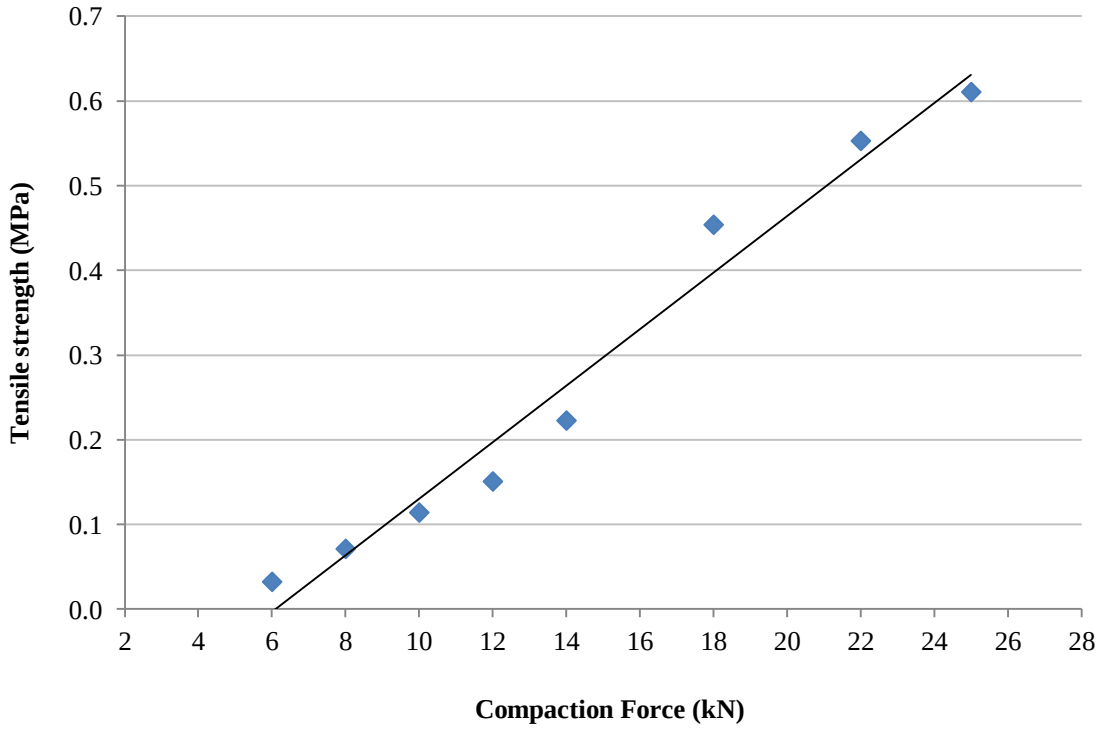


Figure 5. 1 Tensile strength (MPa) of single component Milled 3441 tablets compressed at various compaction forces (kN) at a speed of 30 mm min^{-1}.

This poor mechanical strength of yeast granules was also observed by Al- Mohizea et al. (2007), who tested 26 formulations using dried yeast granules (strain not stated) by various methods including direct compression, wet and dry granulation. In the study it was found that regardless of the good flow properties of yeast granules (Angle of repose of 28° and Carr's index of 5.5%), the compression of granules alone by direct compression technique, was not feasible since the produced tablets showed elastic deformation and cannot withstand handling, confirming the results found here (Al- Mohizea et al. 2007).

As a result of the poor compression properties of Milled 3441 dried yeast granules, compressible excipients MCC and DCP were included as outlined in Chapter 3, Section 3.5. The influence of the amount of MCC in the formulation on the tensile strength was investigated, Table 5.1 (introduced in Chapter 3, Table 3.5).

Table 5. 1 Probiotic tablet formulations with varying proportions of Microcrystalline Cellulose (MCC) and Dibasic Calcium Phosphate (DCP) with a constant amount of Magnesium Stearate (Mg St).

Formulation Number	Amount of Excipient (%(w/w))				Mean Tensile Strength (MPa)
	Milled 3441 yeast	MCC	DCP	Mg St	
5	50	0	49.25	0.75	0.7 ± 0.1
6	50	12.31	36.94	0.75	1.1 ± 0.2
7	50	25	24.25	0.75	1.4± 0.1
8	50	36.94	12.31	0.75	1.5 ± 0.2
9	50	49.25	0	0.75	1.2 ± 0.1

An increase in tensile strength from 0.70 ± 0.04 MPa to 1.48 ± 0.08 MPa was observed as the proportion of MCC in the excipient formulation increased from 0 to 37%(w/w), with a concomitant decrease in the amount of DCP. However, a formulation consisting of 49.25%(w/w) MCC (Formulation 9, Table 5.1) resulted in a decrease in tablet strength from the largest value (Figure 5.2).

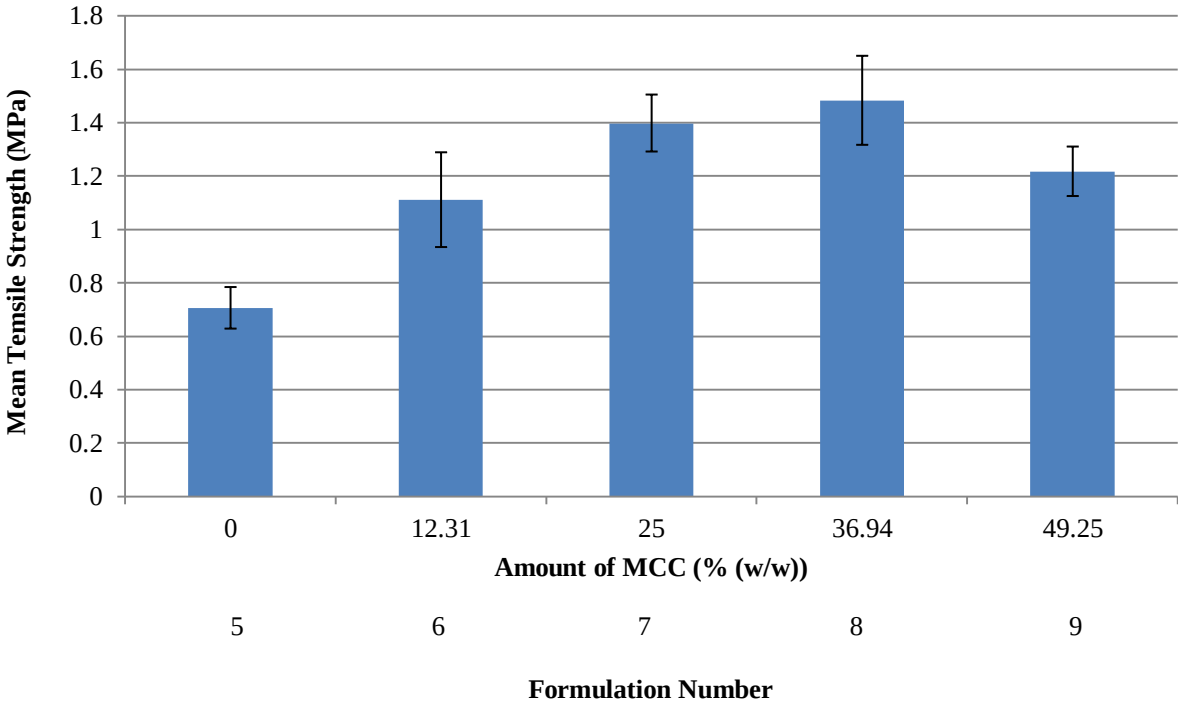


Figure 5. 2 Mean tensile strength of dried yeast tablets containing various proportions (%(w/w)) of MCC and DCP (outlined in Chapter 3, Section 3.5.1 Table 3.1) produced at a compaction force 14 kN (pressure ca. 105 MPa) and speed of 30 mm min^{-1}. Mean ± CI 95% (n=3).

Clearly, a blend of MCC and DCP resulted in tablets with greater tensile strength than tablets of MCC or DCP alone, due to the mixture of compaction behaviour of particles during tabletting, plastic and brittle (fragmentation), respectively. Further evidence of this was reported by Jivraj et al. (2000), who reviewed excipients used for direct compression and found that DCP works best when combined with MCC or starch. No significant difference in

tensile strength (*p>0.05*) was seen between Formulations 7 and 8 and either might have been used for further studies. However, due to the probiotic nature of this product, the higher proportion of DCP incorporated in Formulation 7 compared to Formulation 8 is favourable, because it provides added benefit to the tablet as a source of calcium in nutritional supplements (Rowe et al. 2009). Formulation 7 was therefore studied further.

5.2.1.2 The effect on cell viability

As shown in Figure 5.3, combining powder excipients MCC and DCP within a yeast tablet formulation (Formulations 7 and 8 from Table 5.1) is beneficial regarding cell viability, compared to tablets of MCC without DCP (Formulation 9) as a significantly higher (*p<0.05)* cell viability count was obtained in the former two formulations.

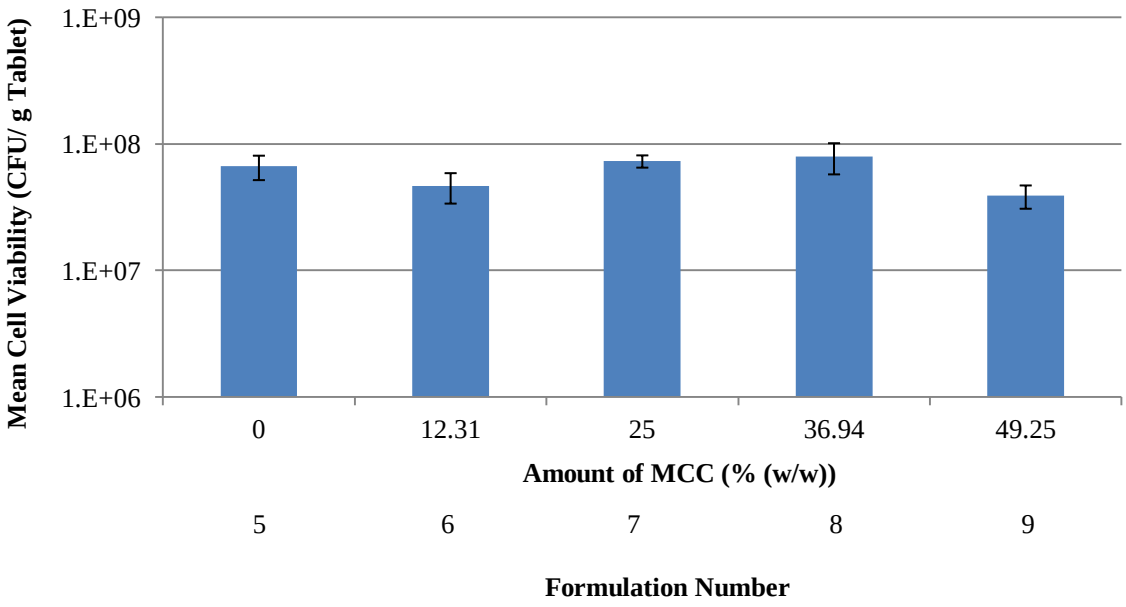


Figure 5. 3 Mean cell viability of dried yeast tablets containing various proportions (%(w/w)) of MCC and DCP produced at compaction force of 14 kN (pressure ca. 105 MPa) and speed of 30 mm min^{-1}. Mean ± CI 95% (n=3)

The plastic compaction behaviour of MCC during tabletting (Jivraj et al. 2000, Roberts et al. 1987) may be a possible cause of yeast cell loss, as tablets containing DCP which undergo brittle fracture (Jivraj et al. 2000, Roberts et al. 1987), resulted in higher cell viability ($6.6 \times 10^7 \pm 1.5 \times 10^7$ CFU /g tablet, Formulation 5). With no significant difference ($p>0.05$) in cell viability between Formulations 7 and 8, the choice of Formulation 7 comprising of 25 %(w/w) MCC and 24.25 %(w/w) DCP was confirmed.

5.2.2 Varying the yeast: powder blend ratio

The effect of the proportions of yeast and powder excipients on tablet strength and compaction behaviour was investigated. The composition of the excipient blend was fixed in the ratios found in Formulation 7 i.e. 25 MCC: 24.25 DCP: 0.75 (%(w/w)) Mg St. At a compaction force of 105 MPa with the chosen excipient proportions, the amount of yeast (%(w/w)) per 1 g tablet was systematically increased (Chapter 3, Section 3.5.2, Table 3.6) and the tablet strength determined, (using the diametrical compression test) in addition to the cell viability (refer to Chapter 3, Section 3.2.4).

5.2.2.1 The effect on tensile strength

A reduction in tablet tensile strength, from 1.51 ± 0.06 MPa to 0.65 ± 0.03 MPa, was observed as the proportion of yeast increased from 50 to 85 %(w/w) (Figure 5.4). This decrease may be explained by the poor binding properties exhibited by yeast granules compared to powder excipients particles. Tablets containing no yeast (Formulation 10), exhibited a tensile strength of 3.09 ± 0.05 MPa, compared to tablets containing no excipients (Formulation 14) i.e. 0.35 ± 0.01 MPa.

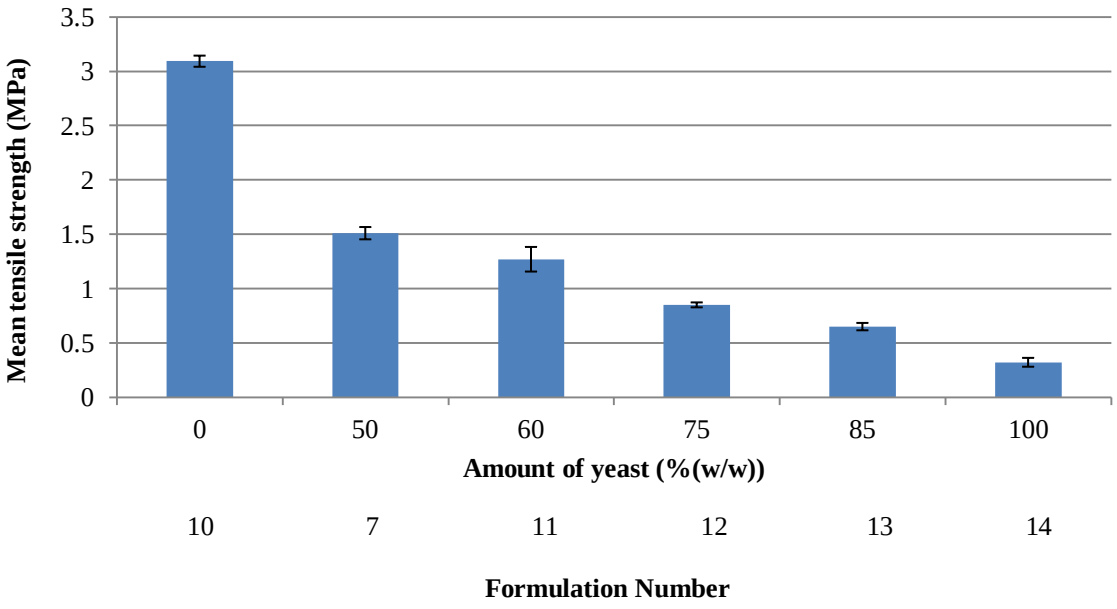


Figure 5. 4 Tensile strength of tablets, produced at compaction force 14 kN (pressure ca. = 105 MPa) and speed of 30 mm min^{-1} containing different proportions (%(w/w)) of dried yeast granules. Mean ± 95 % CI (n=3)

A similar trend was found by Klayraung et al. (2009) when developing bacterial probiotic tablets, where results showed a decrease in tablet strength from 1.2 ± 0.2 N mm^{-2} to 0.42 ± 0.09 N mm^{-2}, when the excipient content was reduced from 89 to 55 %(w/w) (Klayraung et al. 2009). These results highlight the importance of powder excipients and the functionality they exhibit during tablet compaction, not only for yeast granules, but also true for bacterial probiotic tablets. The compaction behaviour of tablets comprising of various proportions of yeast was therefore investigated further.

5.2.2.2 Compaction behaviour analysis

Compaction of powders is widely researched (Mazel et al. 2011) in order to understand the properties of powders under a compaction load. The compaction behaviour of yeast granules, powder excipients and a mixture of both at various concentrations (%(w/w)) were investigated, firstly by conducting the stress strain curves (5.2.2.2.1) and then fit to the Kawakita model (5.2.2.2.2).

5.2.2.2.1 Stress strain curves

Force displacement data obtained during the tabletting of various formulations were used to produce stress strain curves (Figure 5.5).

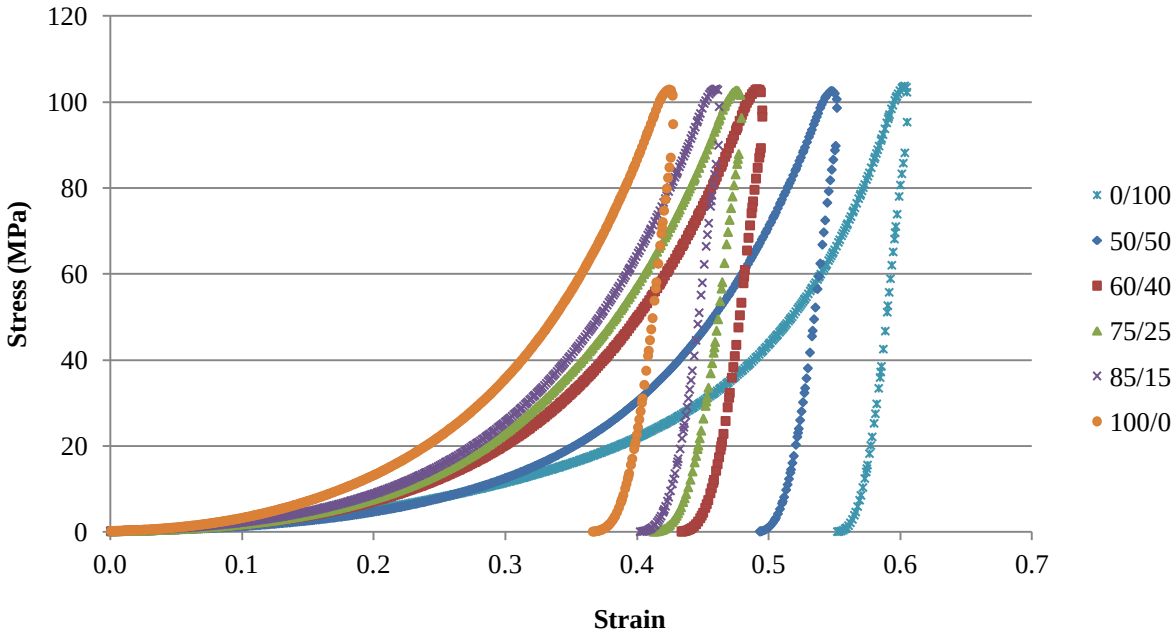


Figure 5. 5 Stress-strain curves for tablets containing varying amount of yeast granules / powder blend (MCC = 25, DCP = 24.25 and Mg St = 0.74 %(w/w)), produced at compaction force of 14 kN (pressure ca.105 MPa) and speed of 30 mm min^{-1}.

It was found that at a given stress, deformation of particles decreased with increasing proportion of yeast. The greatest deformation of 60% was seen with Formulation 10 (tablets not containing yeast), compared to the least deformation (43%) seen with tablets of yeast only (Formulation 14). These results provide further indication of the good binding properties of powder excipients compared to the poor properties of yeast granules. The larger displacement of the powder bed during compaction of powder mixtures can also be used to explain the higher tensile strength values.

The combination of deformation mechanisms (plastic and brittle fracture exhibited by MCC and DCP particles respectively) during the compaction process results in tablets with higher tablet strength. While MCC deforms by increasing the contact area between particles increasing bonding, DCP produces new surfaces resulting in stronger bonding (Roberts et al. 1987; Jivraj et al. 2000). The packing arrangement of the excipient particles around yeast granules can be seen in Figure 5.6. This can be explained by the difference in particle size whereby the smaller excipient particles consolidate around yeast granules and fill the void spaces.

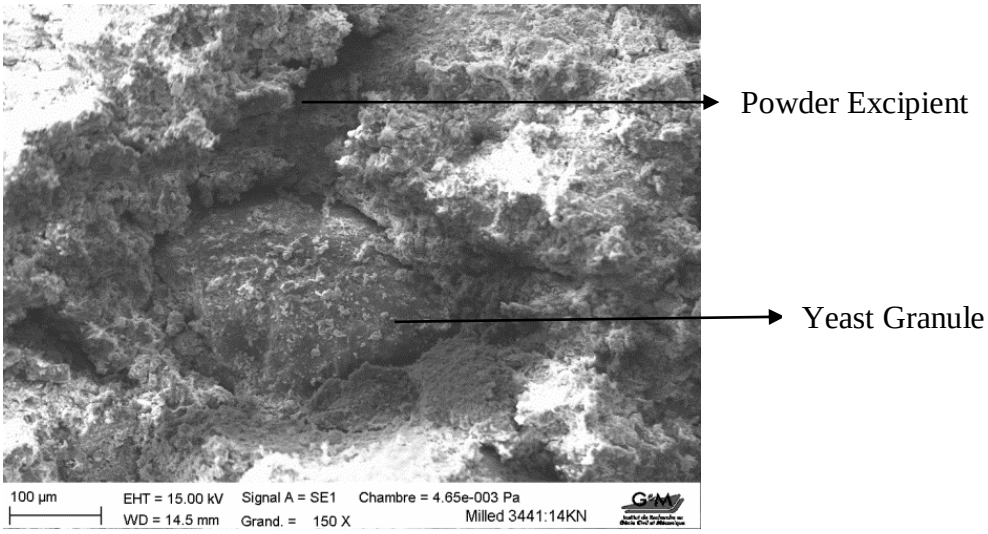


Figure 5. 6 SEM image of the packing arrangement of a tablet consisting of powder excipients and yeast granules in a 50:50 (%(w/w)) ratio (Formulation 7), produced at compaction force of 14 kN (pressure ca. 105 MPa) and speed of 30 mm min^{-1}.

5.2.2.2.2 Fitting the compaction data to the Kawakita model

In order to understand the compression profiles of different formulations, with varying yeast to powder blend ratio, compaction curves were fitted using the empirical Kawakita model (Chapter 3, Section 3.4). The Kawakita equation predicts that there is a linear relationship between σ/ε and σ. The slope and intercept allow the parameters "*a*" and "*b*" to be easily evaluated. Figure 5.7 shows a typical plot for fitting the Kawakita relationship in this linear form, for tablets containing 50 %(w/w) yeast (Formulation 7). A good agreement between the experimental data and the Kawakita model can be seen and this was also found for all other formulations (data not shown).

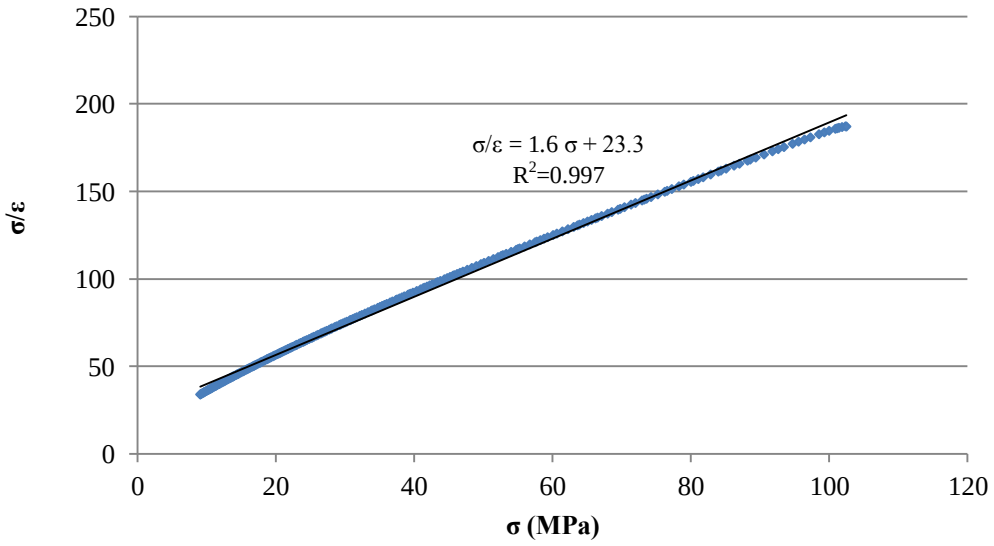


Figure 5. 7 A typical compression curve fitted by Kawakita model for the compaction of tablets containing 50 %(w/w) dried yeast (Formulation 7) produced at a compaction force of 14 kN (pressure ca. 105 MPa) and speed of 30 mm min^{-1}.

The value of the parameter *a* is related to the initial bed voidage and was calculated from the gradient of the slope (Yap et al. 2006). This was found to reduce with increasing amount of yeast, as shown by Table 5.2. Segregation, caused by the difference in particle sizes could explain these results, whereby smaller powder particles are able to fill the voids between larger yeast granules during the die filling process. This initial re-arrangement of particles was also evident after the compaction process, where tablets were produced with powder excipients consolidating around the larger yeast granules.

Constant *b* has the dimension of the reciprocal of stress, which is related to the failure stress of the single particles (Yap et al. 2008). The results show that yeast granules require 34% higher stresses to cause failure than powder blend particles (based on Table 5.2). As previously shown by the stress-strain curves (Figure 5.5), at a given stress, the degree of deformation (strain) for tablets containing pure yeast granules is significantly less ($p<0.05$) than those tablets consisting of powder excipients. As stated by Kawakita (1971), those powders undergoing significant particle rearrangement (at low compression pressures) showed low values of parameter reciprocal *b* and high for parameter *a*, which has been true in this case. The results confirm the good compaction properties of MCC and poor properties of Milled 3441 yeast granules, thus the requirement to be mixed together to produce a tablet strong enough to withstand handling.

Table 5. 2 Kawakita parameters determined from compression of yeast granules and excipients with varying ratio at a compaction force of 14 kN (pressure ca. 105 MPa) and speed of 30 mm min^{-1}. Formulations given in Chapter 3, Section 3.5.2, Table 3.6

Formulation Number	Ratio Yeast: Powder blend (%(w/w))	Kawakita Parameter		*1/b* (MPa)	R^2
		a	*b*		
10	0 : 100	0.66	0.073	13.8	0.999
7	50 : 50	0.60	0.071	13.5	0.997
11	60 : 40	0.55	0.059	16.9	0.996
12	75 : 25	0.53	0.057	17.5	0.995
13	85 : 15	0.52	0.057	17.5	0.995
14	100 : 0	0.49	0.048	21.0	0.996

5.2.2.2.3 Analysis of Kawakita parameters

Kawakita parameters *a* and *1/b* were individually plotted against tensile strength (Figures 5.8 and 5.9, respectively) for probiotic tablets consisting of varying proportions of powder blend and yeast granules (%(w/w)), to determine if a correlation was present. It was found that as the value of initial voidage of powder bed increased, so did the corresponding tensile strength for tablets containing various proportions (%(w/w)) of yeast granules (Figure 5.8). This shows that, despite the initial low porosity after filling the die, tablets resulted in low tensile strength indicating the poor compaction properties of the mixture of particles. The good compaction properties of powder excipients, as previously highlighted, are further confirmed here as formulations containing no yeast granules, with initial high voidage upon filling, still resulted in tablets with higher tensile strength.

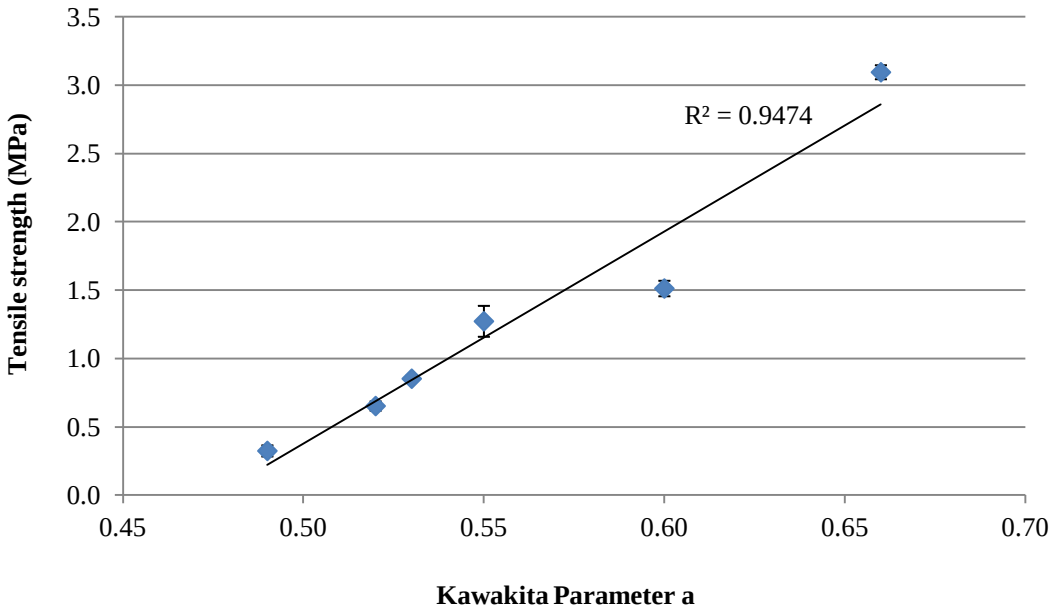


Figure 5. 8 Tensile strength vs. Kawakita parameter a for tablets containing various ratio of yeast: powder blend produced at 14 kN (compaction pressure ca. 105 MPa) and speed of 30 mm min^{-1}.

Kawakita parameter 1/*b* was plotted against tensile strength, as seen in Figure 5.9. A weak correlation (R^2 = 0.65) was observed with *1/b* and tensile strength of resulting tablets. As discussed earlier in Chapter 4, Section 4.2.6.3, the greater the *1/b* value implies less rupture of primary particles during compaction and as a result, fewer fresh surfaces are available for bonding. However, the mechanism of compaction i.e. plastic or brittle deformation needs to be taken into consideration, given the mixture of particles present in the formulation (Frenning et al 2009).

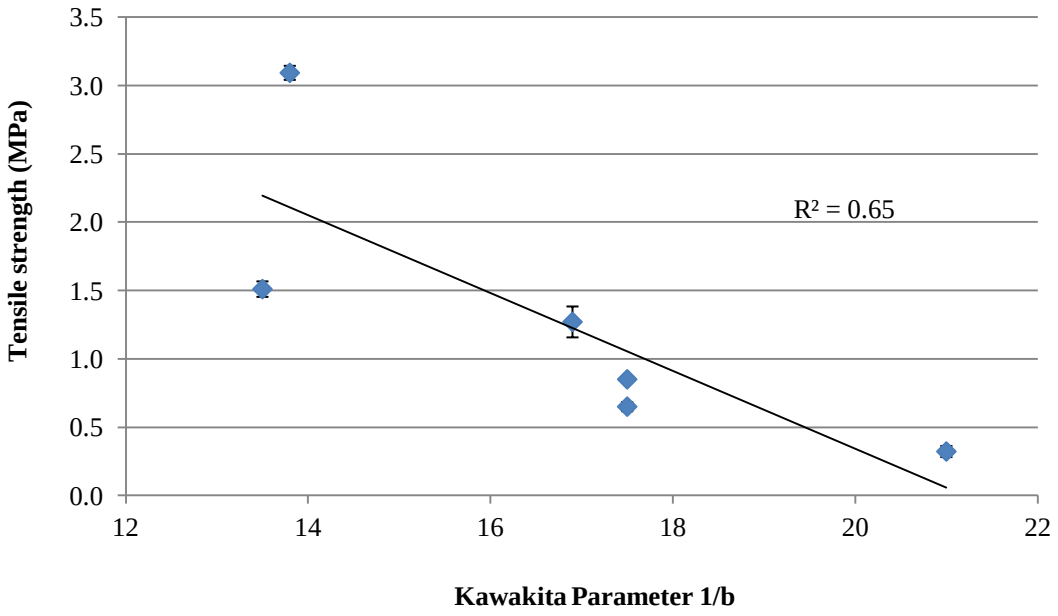


Figure 5. 9 Tensile strength vs. Kawakita parameter *1/b* for tablets containing various ratio of yeast: powder blend produced at 14 kN (compaction pressure ca. 105 MPa) and speed of 30 mm min^{-1}.

5.2.2.3 The effect on cell viability

The effect on cell viability of different proportions of yeast in formulations was also investigated. As seen in Figure 5.10, the number of surviving cells per tablet, was higher when the quantity of yeast in the tablet was increased ($p<0.05$). A similar trend was also found with probiotic tablets containing bacterial strains by Klayraung et al. (2009), where cell viability was found to be higher (7.4 log CFU/ tablet to 8.8 log CFU/ tablet) as the amount of bacteria was increased from 25 to 100 mg, respectively. Studies have been conducted investigating the possible mechanisms for cell viability loss during the powder compaction process.

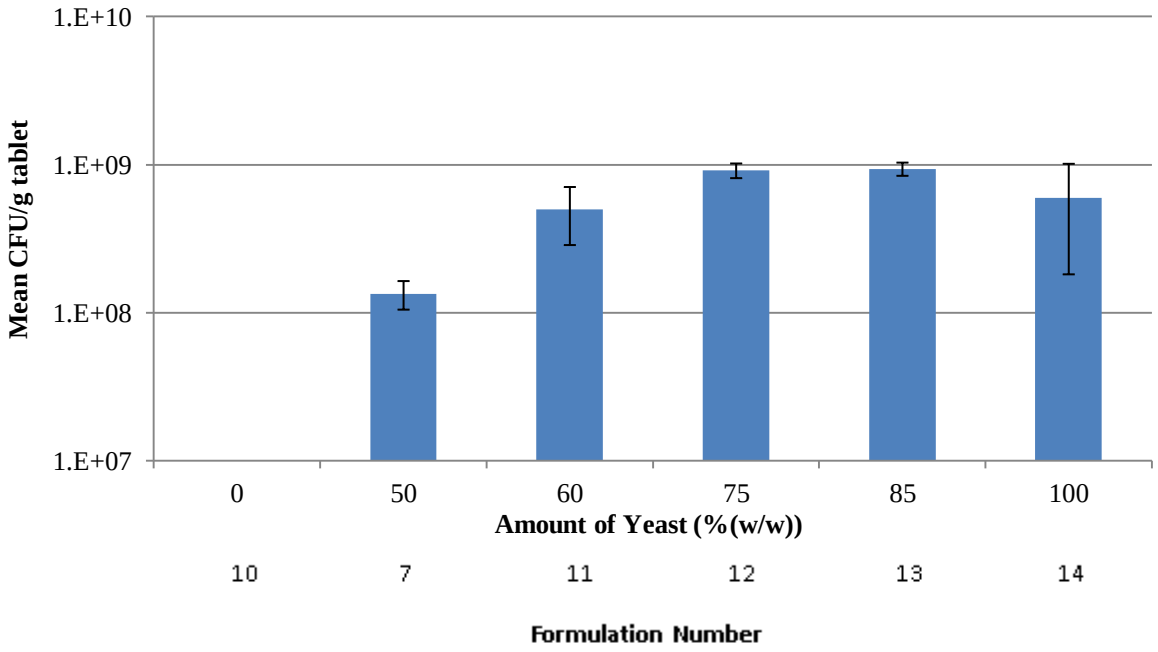


Figure 5. 10 Cell viability of tablets (produced at compression force of 14 kN (pressure ca.105 MPa) containing different proportions of dried yeast granules according to Table 3.6. Mean ± 95 % CI (n=3)

Plumpton (1986) found that the initial stages of compaction (particle rearrangement into void spaces) did not contribute to cell damage, however, it is at the further consolidation stage, when greater pressure is applied, that cell destruction occurs. The suggested mechanisms for cell damage occurring when particles undergo fragmentation or exhibit plastic behaviour are localised "hot spots", shearing forces or pressure itself. However, Plumpton (1986) also found different survival patterns for various yeast particle sizes concluding that pressure alone could not be the cause of cell viability loss during compaction. Based on this, the wide particle size distribution of yeast granules may have contributed in the high viability loss seen with tablets produced in this chapter. For future work, sieving yeast granules could be included as an additional stage before compaction, to determine the impact compaction pressure and size of yeast granule has on the cell viability of resulting tablets.

Overall, Formulation 12 (comprising of 75 %(w/w) Milled 3441 yeast and 25 %(w/w)) exhibited tablets where the cell viability reached desirable amounts to exhibit a health benefit on the host (taken here as 2 $x10^9$ CFU /g tablet per 3 times dosage as set by Lesaffre), thus the corresponding tensile strength is 0.85 ± 0.02 MPa at a compression force of 14 kN (pressure ca. 105 MPa).

5.2.3 The effect of increasing the compaction pressure on tablet properties

Upon the selection of an optimum formulation at the pre-chosen compaction pressure (105 MPa) and speed (30 mm min^{-1}), the effects of compaction pressure on the tensile strength and cell viability of tablets of this specific formulation were investigated. The use of a compaction force of 14 kN (pressure ca. 105 MPa) during the formulation development process was selected based on previous research related to the direct compaction of probiotic microorganisms, discussed in section 5.1.

5.2.3.1 The effect on tensile strength

The slope of a compaction-force versus tensile strength profile provides qualitative information about the ability of material to produce strong tablets (Jain 1999). It was found that, for a given formulation containing 75%(w/w) Milled 3441 and 25%(w/w) powder blend, Formulation 12 (Chapter 3, Table 3.6), as the compaction pressure increased from 95 to 150 MPa, the tensile strength of tablets increased by 61% (Figure 5.11). This trend was also observed by Klayraung et al. (2009), who found that the tensile strength of tablets each containing 100 mg probiotic bacteria and 125 mg HPMCP 55, increased from 0.09 to 2.75 MPa when the compaction force increased from 2 to 20 kN, respectively. Pitt et al. (2013) also observed this increasing trend, when investigating a direct compression formulation consisting of an active ingredient, MCC and other pharmaceutical powders with magnesium stearate as the lubricant.

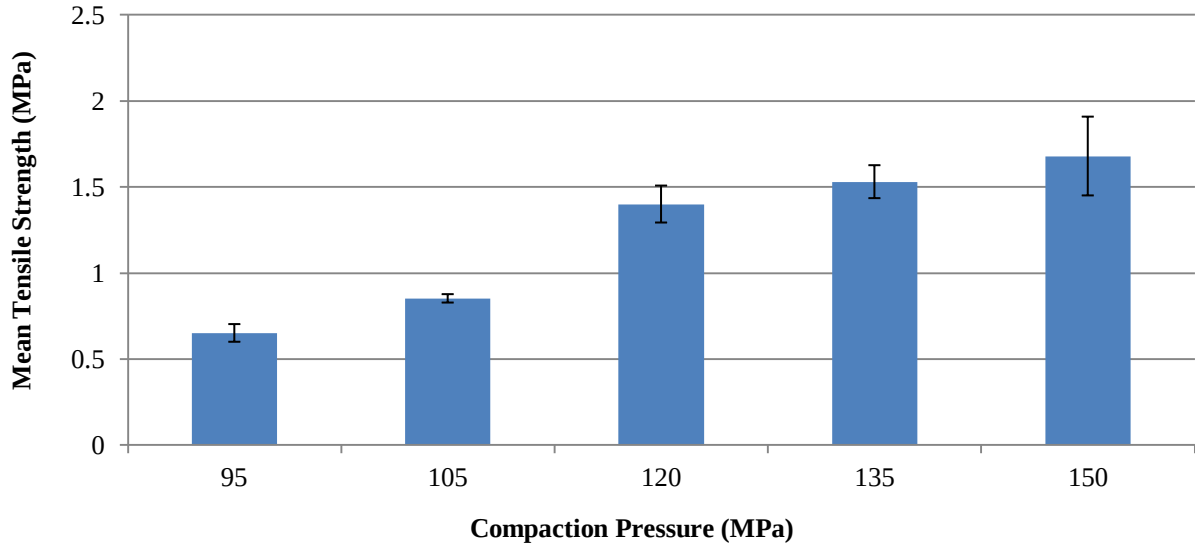


Figure 5. 11 Tensile strength of yeast probiotic tablets (produced at compaction pressure 105 MPa) produced according to Formulation 12 (Chapter 3, Section 3.5.2, Table 3.6). Mean ± 95 % CI (n=3)

Although it may seem that obtaining a higher tensile strength from increasing the compaction force may be beneficial, it must be considered that steeper gradients on a tensile strength versus compaction pressure curve, may suggest potential problems in production processes, as a small change in the compression force could cause significant increases in the tablet crushing strength which could result in capping (Jain et al. 1999). In this case, selecting the initial compaction pressure of 105 MPa compaction pressure was confirmed and remained fixed for further studies.

5.2.3.2 The effect on cell viability

The effect of compaction pressure on yeast cell viability for a given formulation (Formulation 12) was also investigated. It was found, as seen in Figure 5.12, that as the compression pressure increased from 105 to 150 MPa, the cell viability reduced significantly (p<0.05). Moussa et al. (2013), suggesting that damage occurring at the cellular level, such as membrane permeabilisation, would occur when cells undergo sufficient volume compression, i.e. mechanical stress.

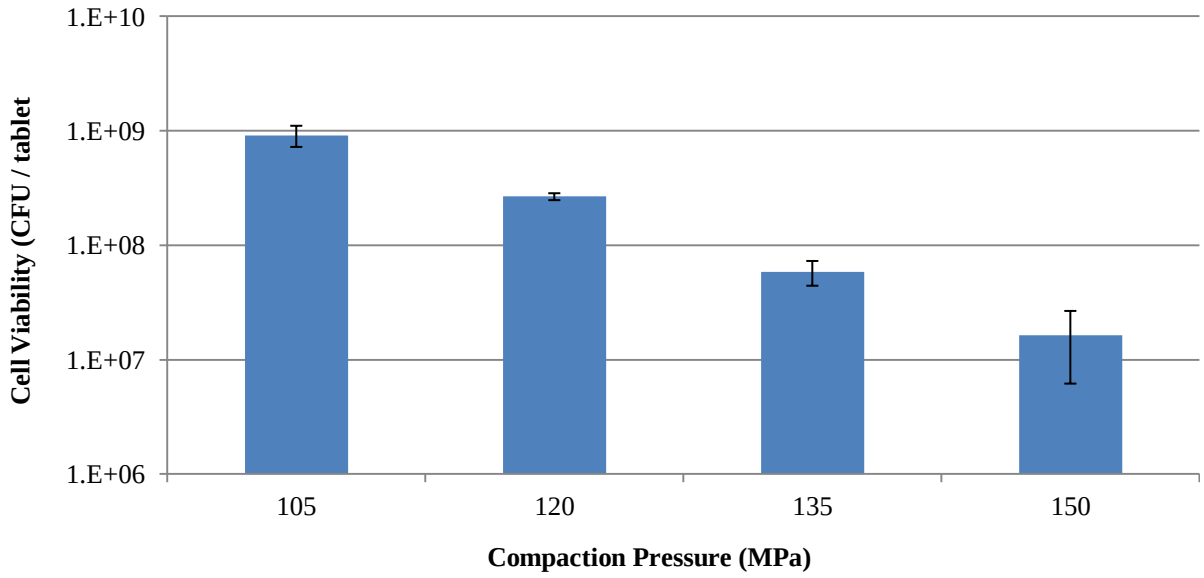


Figure 5. 12 Cell viability of yeast probiotic tablets (produced at compaction pressure 105 MPa) produced according to Formulation 12 (Chapter 3, Section 3.5.2, Table 3.6). Mean ± 95 % CI (n=3)

This could be a possible explanation for the loss of cell viability during compaction. It has also been stated by Ludwig et al. (2002), that pressure may induce severe compressive stress on the cell wall, entailing the denaturation of membrane-bound proteins. However, this trend seen with probiotic yeast has not been found to be true for tablets containing probiotic bacteria. Klayraung et al. (2009) found that as the compression force increased from 2 to 20 kN, the survival of lactic acid bacteria (%) in tablets, increased from 82 to 89% which is quite surprising. However, the variation from 89 to 82% is less than 10%, thus the significance of these results and conclusions are questioned. This indicates that there may be large differences in probiotic microorganisms, especially bacterial and yeast cellular behaviour during the compaction process.

In order to produce a tablet exhibiting the required yeast dosage for probiotic effects (2 x 10^9 CFU /g tablet per 3 times dosage), it seems a compromise between tensile strength and cell viability is essential. Increasing the compaction force resulted in stronger tablets with higher tensile strengths (as seen in Section 4.5.1) but the resulting cell viability for that high compaction force was reduced significantly. As the main purpose of this tablet is the probiotic nature, the main priority is to ensure adequate yeast cell viability is present for their probiotic functionality. In conclusion, the selected force for tablets containing 75 %(w/w) yeast and 25 %(w/w) powder blend was 105 MPa.

5.2.4 Speed-Sensitivity test

5.2.4.1 The effect of compaction speed on tensile strength

Compression speed can have significant effects on the compaction properties of pharmaceutical powders. This is a challenge during scale up and technology transfer, where tabletting speeds are significantly increased, hence needs to be considered and tested during initial formulation development stages (Tye et al. 2005). Tablets containing the selected Formulation 12 were produced at various compaction speeds and tested for their mechanical properties. A significant decrease (p<0.05) in mean tensile strength was observed as the compaction speed increased from 1 to 60 mm min^{-1}, as shown in Figure 5.13.

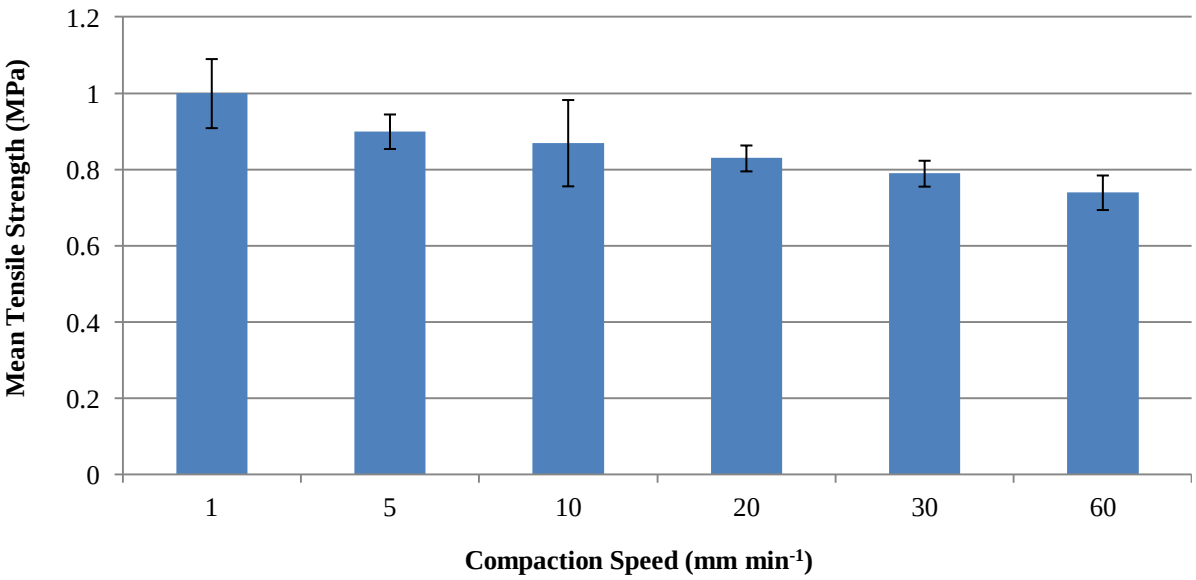


Figure 5. 13 The mean tensile strength of tablets produced at varying compaction speeds according to formulation 12 (Chapter 3, Section 3.2.2, Table 3.6) Mean ± 95% CI

Tablet press on a commercial scale would occur at higher speeds than those used in this test, thus these results provide a predictive indication of the effects speed can have on tablet performance. It is believed that this time dependency arises from when the load is removed (ejection stage) after compaction for materials undergoing primarily plastic deformation (Tye 2005). This could be true for materials like MCC. However, compaction of brittle materials (such as DCP) is less speed dependent because fragmentation is rapidly achieved and prolonged exposure to the force has a more limited effect on tablet properties (Tye et al. 2005). As the powder blend comprises of a mixture of both brittle and plastic exhibiting powders, the effect of speed changes in the range of 5 to 30 mm min^{-1}, did not seem to affect the tensile strength. Producing tablets at 60 mm min^{-1} would result in tablets with reduced tensile strength and selecting 1 mm min^{-1} would result in a slow production time, hence 30 mm min^{-1} was selected as the compaction speed for the production of tablets containing dried probiotic yeast. The selection of a compaction speed of 30 mm min^{-1} is further explored in the following section.

5.2.4.2 The effect of compaction speed on cell viability

As seen by Figure 5.14, speed did not have an effect on the cell viability post tabletting. This indicates that cell viability loss during the compaction process could be due to the pressure upon yeast granules regardless of the speed. These results also indicate that no or little effect on the cell viability could occur during scale up production. Further research would be required in order to investigate the full effects of scale up, as mentioned later in Chapter 7.2. As discussed above, taking into account production efficiency, a compaction speed of 30 mm min^{-1} was selected.

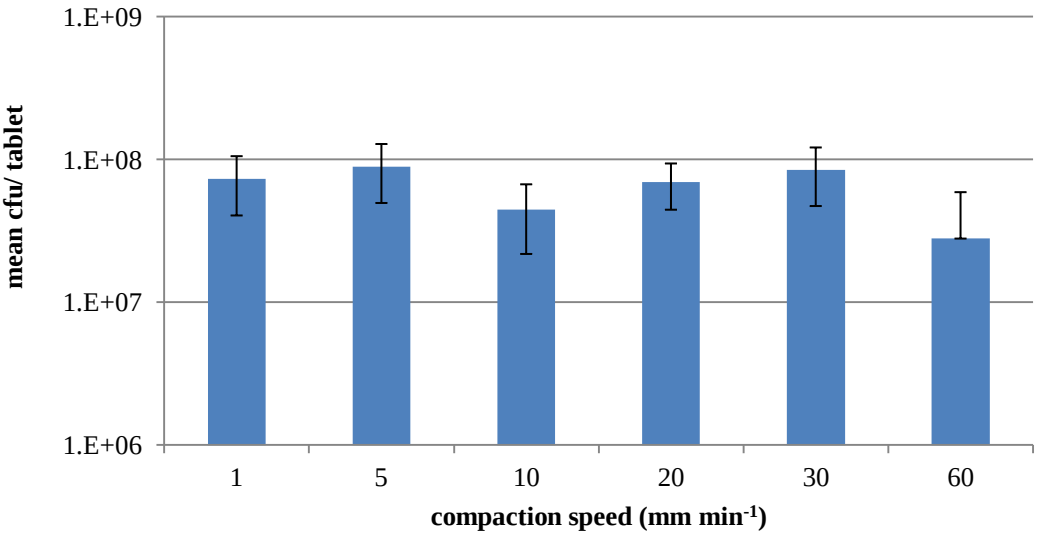


Figure 5. 14 Mean cell viability of tablets produced at varying compaction speeds according to formulation 12 (Chapter 3, Section 3.2.2, Table 3.6) Mean ± 95% CI

5.3 CONCLUSION

In this chapter, the formulation of probiotic tablets containing dried Milled 3441 yeast granules was optimised. Initially, the composition of the powder blend was determined by testing various proportions (%(w/w)) of MCC and DCP into tablets. The selected formulation (Formulation number 7) comprised of 50% milled 3441 yeast, 25% MCC and 24.25 % DCP, with 0.75 %(w/w) Mg St as the lubricant. Upon this selection, the ratio of Milled 3441 to powder blend was increased sequentially, to determine the optimum tablet exhibiting adequate cell viability (2 $x10^9$ CFU/ g per 3 times dosage) and tensile strength. The compaction behaviour was also analysed in order to form a better understanding of the compaction behaviour of powder particles and yeast granules. The poor binding properties of yeast granules and importance of including a binder and filler in a yeast probiotic tablet formulation was highlighted, as tablets with increasing amount (%(w/w)) of yeast exhibited decreasing tensile strength.

The selected Formulation was 12, containing 75 %(w/w) yeast granules and 25 %(w/w) powder blend based on tablets exhibiting an adequate tensile strength of 0.85 ± 0.02 MPa and cell viability of 9.14 $x10^8$ ± 0.5 x 10^8 CFU /g tablet. These results present the opportunity to administer dried yeast with probiotic functionality in a tablet oral dosage form.

Processing conditions such as compaction force and speed were also investigated for tablets comprising of Formulation 12. It was found that increasing the compaction pressure from 105 to 150 MPa, resulted in tablets with increasing tensile strength (MPa), however had an adverse effect on cell viability (which decreased significantly). Speed, on the other hand, was seen to have a significant (p<0.05) effect on tablet strength. A decrease in mean tensile

strength was observed as the compaction speed increased from 1 to 60 mm min^{-1} with no significant effect on the cell viability.

Tablets of the selected formulation for probiotics (comprising of 75 %(w/w) Milled 3441 granules and 25 %(w/w) powder blend) were produced at a compaction pressure of 105 MPa and speed of 30 mm min^{-1}, resulting in tablets with tensile strength of 0.85 ± 0.02 MPa and cell viability of 9.14 x 10^8 ± 0.5 x 10^8 CFU /g tablet. At this stage, this is considered as the best formulation with regards to cell viability because it meets the specification set by Lesaffre; cell viability of 2 x 10^9 CFU in three dosages based on 1 g tablets. However, the tensile strength did not meet the criteria of 1 MPa.

CHAPTER 6: THE APPLICATION OF A LEADING FORMULATON TO NEW YEAST SAMPLES AND THE STUDIES OF FRIABILITY, DISSOLUTION AND STORAGE STABILITY

6.1 INTRODUCTION

A protocol formulation of powder excipients (MCC 25 % w/w, 24.25 %w/w DCP and 0.75 %w/w Mg St) and 50 %w/w dried yeast granules (Milled 3441) was developed as discussed in Chapter 5. Moving forward, this chapter explores applying the protocol formulation to a variety of yeast granules produced with different coating materials. Upon the selection of a final formulation, is it important to conduct tests to ensure the final product is suitable for consumer/patient use. To be released on the market, pharmaceutical products need to comply with Pharmacopeia standards. Post production, tablets are exposed to bulk handling, transport and other post-compaction operations during which the dissolution profile and mechanical integrity must be maintained (Sinka et al 2009). It is mandatory to assess the capability of providing cost-effective, industrial-scale quantities of probiotic strains for clinical and/ or commercial (i.e. the industrial feasibility) (Piano et al. 2006). This is also the case for tablets containing probiotic yeast.

The purpose of stability testing, as defined by the U.S. Department of Health and Human Services Food and Drug Administration (2002) is to "provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to establish a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions". From obtaining this information, the type of packaging required during storage

of the probiotic yeast tablets (such as blister packs, amber bottles) can be determined. The effect of temperature and humidity on the final product (especially those containing microorganisms) is of great importance and is required to be investigated as these environmental conditions can alter the quality of the final product, altering its functionality. An increase in relative humidity can also negatively impact tablet strength (Thoorens et al. 2014). Moisture content related to water activity, is also a parameter that can influence particles and overall tablet properties. A recent review by Thoorens et al. (2014) discusses the influences of moisture content of MCC on compaction properties, tensile strength, and viscoelastic properties. According to reports received by Lesaffre (Human Care & Food Additives, France), a 1 log loss was observed in cell viability of Actisaf STD yeast granules when yeast samples were exposed to thermal stress for 2 minutes in dry heat conditions (85°C, 7% moisture content and 90°C, 6% moisture content). It was also reported that those yeast samples exposed to high levels of humidity (90% RH) had visible swelling of the granule surface.

For this project the water activity (a_w) was not measured and taken under the assumption that the tablet properties (mechanical and cell viability) would not significantly change. The effect of relative humidity and/ or water activity of pharmaceutical powders and yeast granules are factors to consider for future work.

The release of tablets is of high importance in determining the time taken for yeast granules to be released at the intended site of action (small intestine), thus exerting their probiotic functionality. In addition reproducibility of a selected formulation is vital to ensure the cell viability is reasonably consistent across each tablet.

The work described in this chapter involves new yeast samples provided by Lesaffre, which were prepared with different coating materials and compositions, as outlined in Table 6.1. The new coated yeast granules were produced and intended for Lesaffre's additional business projects. As part of the preliminary work for this chapter, these yeast samples were tested for their total viable cell count, compressed into tablets and characterised for their mechanical strength and resulting cell viability (according to the methods outlined in Chapter 3, Sections 3.2.2, 3.2.3 Table 3.2 and 3.2.4, respectively). The defined formulation and processing conditions (compression pressure 105 MPa and speed of 30 mm min^{-1}) from Chapter 5 were applied to these samples and from this, lead formulations were identified. Further tests such as friability, dissolution and storage stability tests were conducted in order to determine their suitability for patient and/ or consumer use, in compliance with Pharmacopeia standards. A recommended yeast sample is provided, indicating its properties with regards to cell viability and tensile strength post tabletting.

Table 6. 1 Detailed list of new yeast granule samples received from Lesaffre (The code names of samples are those provided by Lesaffre)

	Description
Wax coated M	Actisaf STD coated: 35% coating in mass (25% carnauba wax + 10% soya oil), coating consists of double layers (layer 1 is carnauba wax, layer 2 is soybean oil).
Wax coated P	Actisaf STD coated: 25% coating in mass consisting of one monolayer comprising 2 materials; 3.9% glycerol monostearate and 20.3% vegetable fat from palm/coco and sunflower.
Lipid coated samples:	Instant active dried yeast (vermicelli shaped) coated with various lipids in various compositions.
Lipid coated MG01	Witosan 42/44 (15.1% of coating)
Lipid coated MG02	Acetem 19.4% of coating
Lipid coated MG03	Mono Stearate Glycerol (MSG) 20% of coating
Lipid coated MG05	Colza oil + MSG (30 + 5% of coating)
Lipid coated MG07	Soya oil+ MSG +calcium stearate (25+ 8+ 2% of coating)

6.2 RESULTS

6.2.1 Preliminary results

Based on the enumeration of total yeast living cells of coated yeast granules, little difference in the cell viability of wax coated and various lipid coated yeast was found (Figure 6.1).

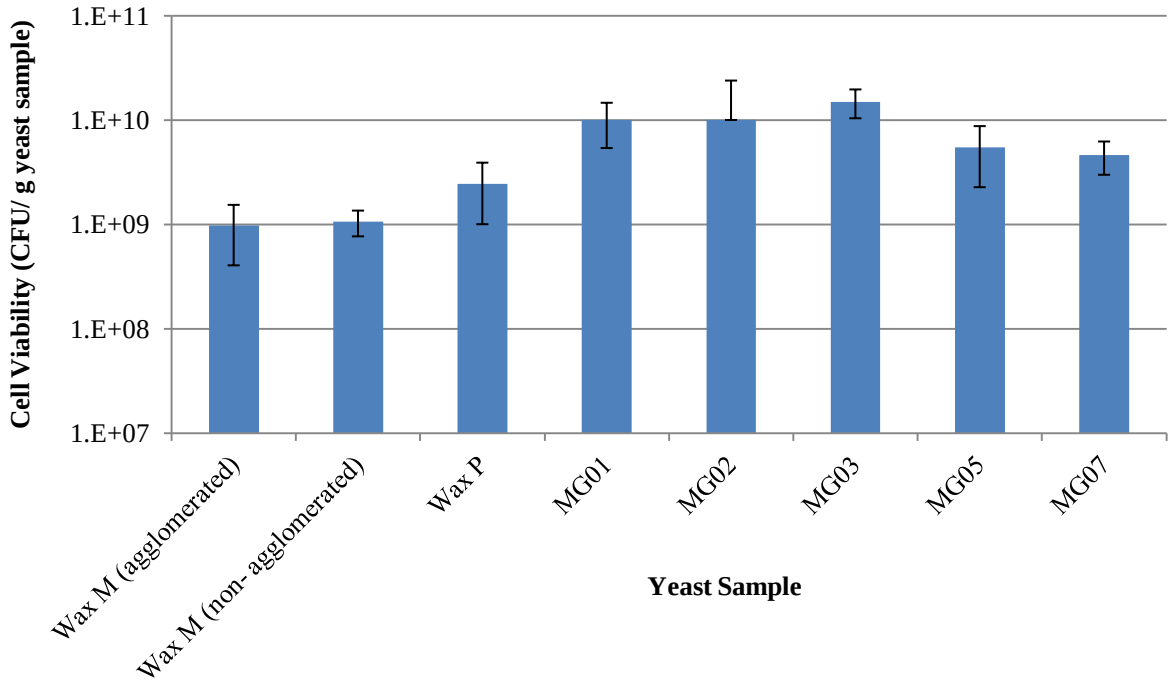


Figure 6. 1 Enumeration of total yeast living cells for new dried yeast samples (described in Table 6.1) supplied by Lesaffre (France. Mean ± 95% CI (n=3)

This is based on the variability of viability across the sample range. Tablets were produced and tested for their tensile strength (Figure 6.2). The results highlight the good compaction properties of Wax M (non- agglomerated) and MG03 granules. However, the errors need to be considered and may be due to the variability of granules during production, since the purpose of these samples (supplied by Lesaffre, France) was mainly for development and controlled methods from the company had not yet been fixed.

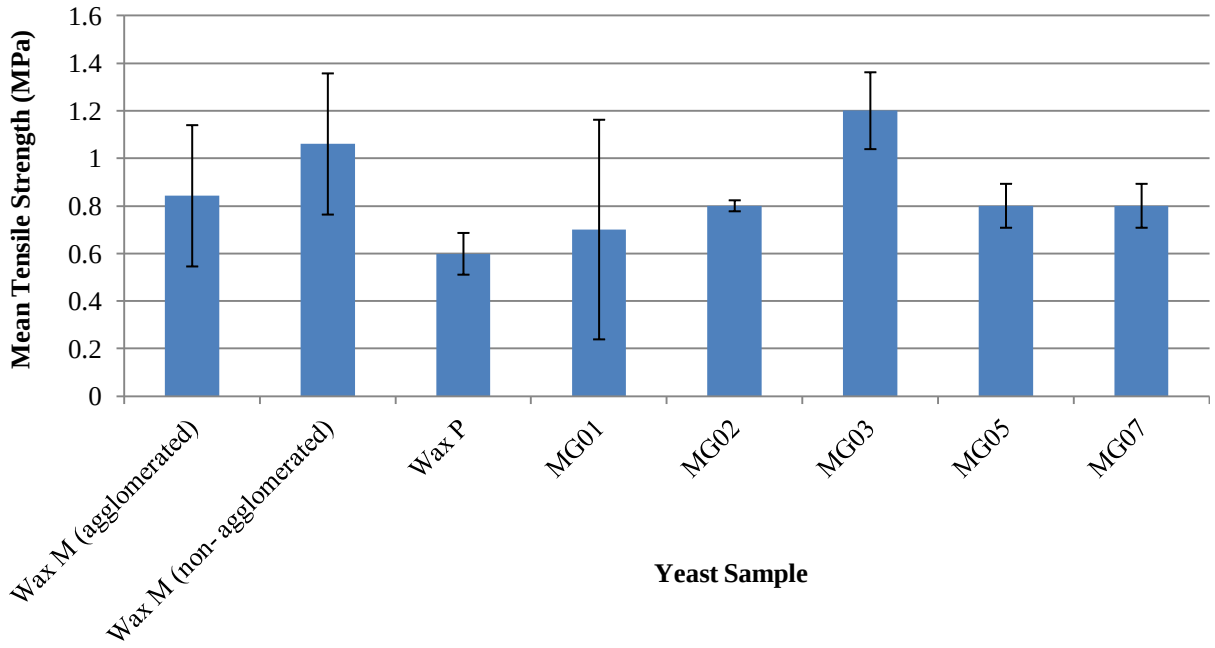


Figure 6. 2 Mean tensile strength (MPa) of probiotic tablets comprising of new yeast samples (outlined in Table 6.1) produced according to Table Chapter 3, Table 3.2 using 14 kN compaction force (ca. 105 MPa compaction pressure) and speed of 30 mm min^{-1}. Mean ± 95% (n=3)

The cell viability of resulting tablets was also determined and as seen in Figure 6.3, Wax P resulted in a significantly higher ($P<0.05$) mean cell viability value compared to other wax and lipid coated yeast granules. Based on these results, tablets containing Wax M (non agglomerated), P and MG03 were selected as lead formulations and are further discussed below (Section 6.2.2).

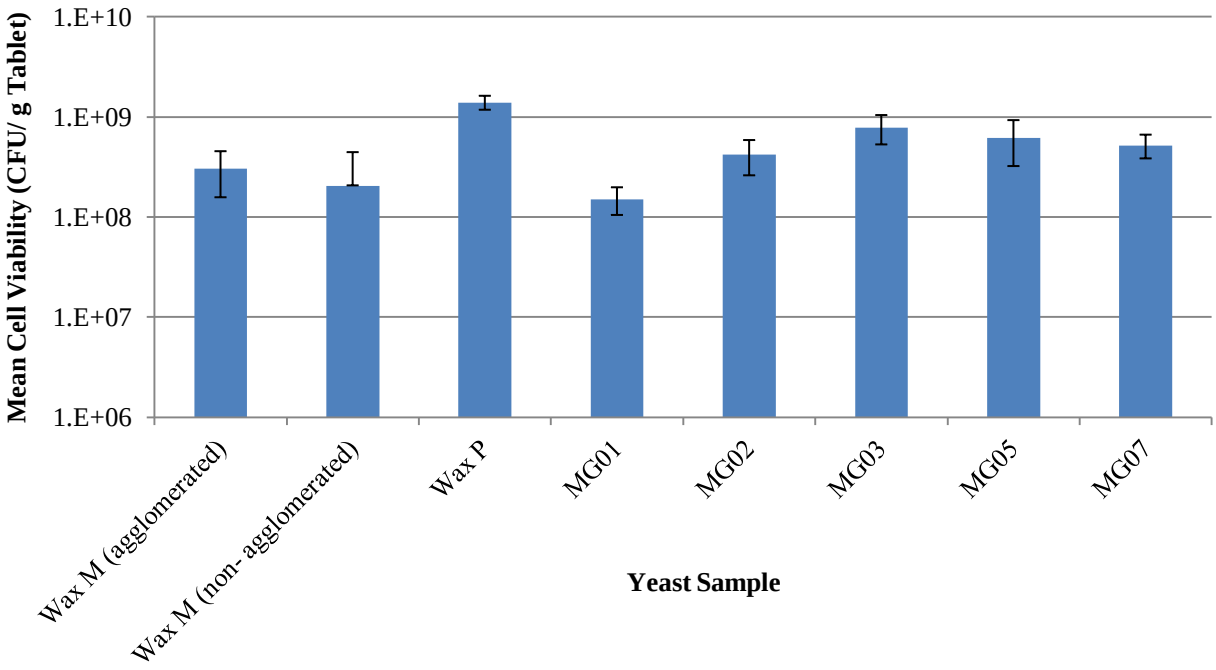


Figure 6. 3 Mean cell viability (CFU /g tablet) of probiotic tablets comprising of new yeast samples (outlined in Table 6.1) produced according to Table Chapter 3, Table 3.2 using 14 kN compaction force (ca. 105 MPa compaction pressure) and speed of 30 mm min^{-1}. Mean ± 95%

6.2.2 Lead formulations

The formulations that are considered as "lead" formulations are those where the tensile strength and/or cell viability values meet the required specification; here set by Lesaffre (Human Care and Feed Additives (France)) to be 1 MPa and 2 x 10^9 CFU /g in 3 dosages (tablets). Table 6.2 identifies the yeast samples and the corresponding formulations which resulted in tablets meeting one aspect of the specification indicated by the green shaded cells.

Table 6. 2 Results of those cells shaded in green represents lead probiotic formulations of various yeast samples provided by Lesaffre Human Care and Feed Additives (France) (Compaction speed 30 mm min^{-1} and compaction force 14 kN)

Key:
*** = compaction speed of 1 mm min^{-1}** **^ = compaction force of 16 kN**

Yeast Sample	Formulation yeast : powder blend (% w/w)	Tensile Strength (MPa)	Cell viability (CFU /g tablet)	CFU per three tablet dose
Milled 3441	50: 50	**1.3 ± 0.1**	2.8 x10^{8} ± 0.3 x 10^{8}	8.4 x 10^{8}
	60: 40	**1.3 ± 0.1**	4.96 x 10^{8} ± 2.10 x10^{8}	1.5 x 10^{9}
	75: 25	0.85 ± 0.02	9.14 x10^{8} ± 1.04 x10^{8}	**2.7 x 10^{9}**
	85: 15	0.65 ± 0.03	9.36 x10^{8} ± 0.96 x10^{8}	**2.8 x 10^{9}**
	100: 0	0.35 ± 0.04	5.96 x 10^{8} ± 4.16 x10^{8}	**1.8 x 10^{9}**
	75: 25 *	**1.00 ± 0.09**	7.32 x10^{7} ± 3.26 x10^{7}	2.2 x 10^{8}
Wax M	50: 50	**1.1 ± 0.1**	2.05 x10^{8} ± 2.3 x10^{8}	6.2 x 10^{8}
Wax P	50: 50	0.6 ± 0.1	1.09 x10^{9} ± 1.05 x10^{9}	**3.3 x 10^{9}**
	50: 50^	0.6 ± 0.2	7.82 x10^{8} ± 1.49 x10^{8}	**2.4 x 10^{9}**
MG03	50: 50	**1.4 ± 0.1**	1.15 x10^{8} ± 0.44 x10^{8}	3.5 x 10^{8}

All tablets were produced and tested according to methods outlined in Chapter 3, using a compaction speed of 30 mm min^{-1} (except * which was produced at 1 mm min^{-1}) and a compaction force of 14 kN (pressure ca. 105 MPa), except ^ which was produced at 16 kN. Due to the high cell viability results obtained with Wax P (using Formulation 7; 50%(w/w) yeast: 50%(w/w) excipient), an attempt to increase the tensile strength of tablets was made by increasing the compaction force from 14 to 16 kN. However, as the results from Table 6.2 show, increasing the compaction force had no effect on tablet strength. Various tests of tablet properties such as friability, dissolution and storage stability were determined according to the methods in Chapter 3, Section 3.6 and results discussed below.

6.2.3 Friability

Friability test, as defined by the Ph. Eur. (5.0) is intended to "determine, under defined conditions, the friability of uncoated tablets, the phenomenon whereby tablet surfaces are damaged and/or show evidence of lamination or breakage when subjected to mechanical shock or attrition". The strength of tablets is defined or seen as "acceptable" according to their performance in the friability test. No quantitative "pass" criteria for tablet strength are provided by the Pharmacopoeias, thus highlighting the significance and necessity of friability testing. The tablets with the lead formulations listed in Table 6.2 were tested and it was found that only those tablets containing MG03 met the standard (< 1% weight loss). As a result, this formulation, comprising of 50:50 %(w/w) yeast: powder blend was selected as the best formulation for probiotic tablets containing dried yeast. Tablets of this formulation exhibited a tensile strength of 1.4 ± 0.1 MPa and contained 1.15 $x10^8$ ± 0.4 $x10^8$ CFU/ g tablet, resulting in a daily dose of 3.5 $x10^8$ CFU /g assuming a three tablet dose.

The relationship between tablet strength and friability was investigated for probiotic tablets containing of various yeast samples (Figure 6.4).

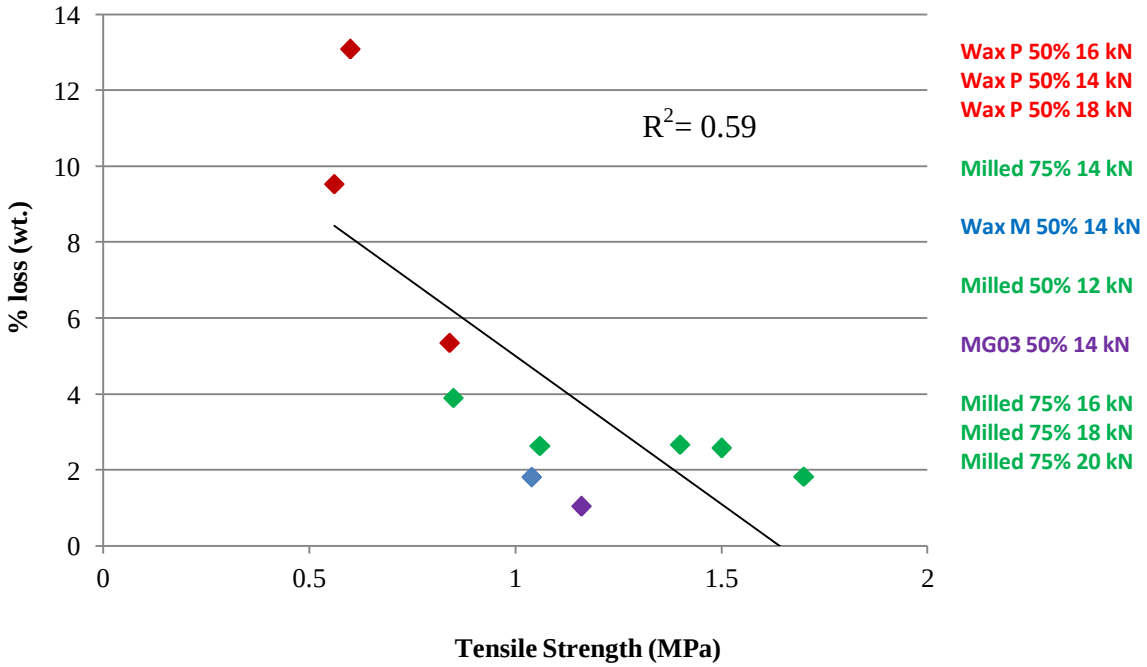


Figure 6. 4 % weight loss from friability results vs. tensile strength for various leading yeast probiotic tablets.

A weak correlation (R^2= 0.59) can be seen between an increase in tensile strength and decrease in % weight loss. This is supported by Riippi et al. (1998) who found an increase of compaction force, causes an increase in the force to crush tablets to failure and as a result a reduction in friability.

The relationship between tablet strength and friability for pharmaceutical tablets is a point of interest (Autamashih 2011), however there is a lack of published research. The results obtained here, indicate that during the initial stages of formulation development, determining the tensile strength is a suitable inverse indicator of friability. However, controlling and

limiting factors of the friability test include the large sample size (10 tablets per test), in addition to the destructive method, which can be an issue especially when active ingredients are not available in large quantities.

6.2.4 Dissolution test of probiotic tablets containing Milled 3441 and MG03 yeast granules

The release profile, and the effect of pH was determined for tablets containing Milled 3441 yeast granules in the ratio of 50:50 %(w/w) yeast: powder blend (Formulation 7) testing at pH 2 and pH 7 (refer to Chapter 3, Section 3.6.1). As shown in Figure 6.5, plateau were reached after 80 min at pH 2 and 40 min at pH 7, indicating a the maximum amount of yeast to be released and rehydrated had been reached.

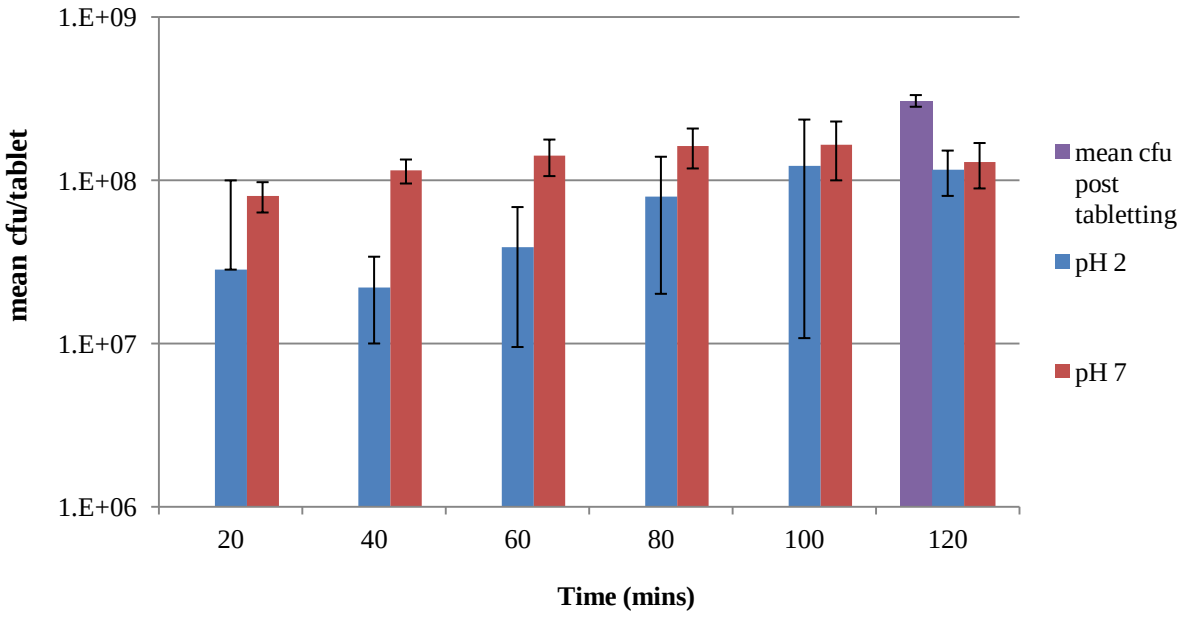


Figure 6. 5 Dissolution profile of probiotic tablets containing 50 %(w/w) Milled 3441 yeast granules: 50%(w/w) powder blend (Formulation 7), produced at compression force of 14 kN (pressure ca. 105 MPa) and speed of 30 mm min^{-1}, using pH 2 and pH 7 medium. Mean ± 95% CI (n=3).

It was observed that at 120 min, no significant difference ($p<0.05$) was seen between yeast released in pH 2 and pH 7 media. This suggests that low pH did not have a detrimental effect on cell viability, in contrast to behaviour reported for probiotic bacteria (Chan and Zhang 2005).

A decrease in the total amount of yeast released ($1.2 \times 10^8 \pm 0.4 \times 10^8$ CFU /g tablet for pH 2 and $1.3 \times 10^8 \pm 0.9 \times 10^8$ CFU /g tablet for pH 7) was found compared to the control value ($3.1 \times 10^8 \pm 0.6 \times 10^8$ CFU /g tablet), which was obtained using a homogeniser. A residue of the disintegrated tablet remained at the bottom of the beaker, showing that not all of the yeast granules were released. In addition, DCP is practically insoluble in water (Rowe et al. 2009) highlighting its limitation. Since the above results confirmed that yeast cells are not sensitive to low pH, a trial dissolution profile for MG03 containing tablets was determined using a pH 7 medium. A plateau of cell release can be seen from 15-20 min when tablets were placed in a medium at pH 7 (Figure 6.6). The results pass the specification outlined by the Ph. Eur. (5.0); therefore the inclusion of a disintegrant is not necessary with these formulations.

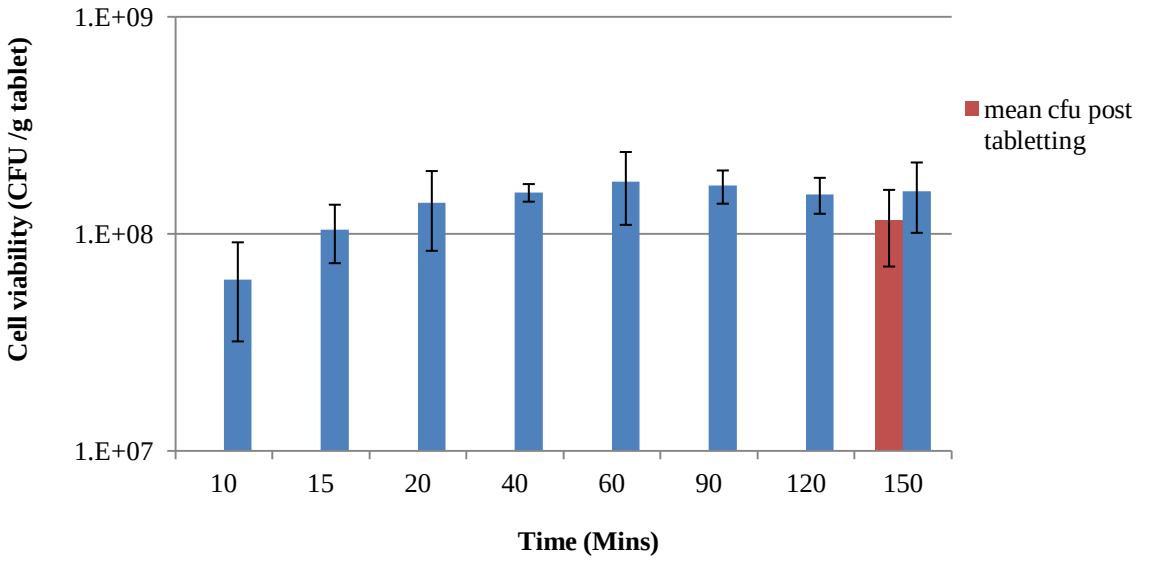


Figure 6. 6 Dissolution profile of probiotic tablets comprising of 50 %(w/w) MG03 yeast granules: 50%(w/w) powder blend (Formulation 7), produced at compression force of 14 kN (pressure ca. 105 MPa) and speed of 30 mm min^{-1}, using pH 7 medium. Mean ± 95% CI (n=3)

6.2.5 Storage stability test

The effect of storage temperature on the cell viability of dried yeast granules and tablets containing such granules is of high importance. It has been reported, that the viability of active dry yeast products is significantly reduced during storage at high temperatures; 40°C for 2 weeks (Sullivan et al 2011). A short term storage stability test was conducted on tablets containing MG03 (according to Chapter 3, Section 3.6.3) and cell viability determined by the homogenisation technique (using an UltraTurrax as outlined in Chapter 3, Section 3.2.2.2). The effect of storage temperature on tablets containing MG03 was investigated and results are shown in Figure 6.7.

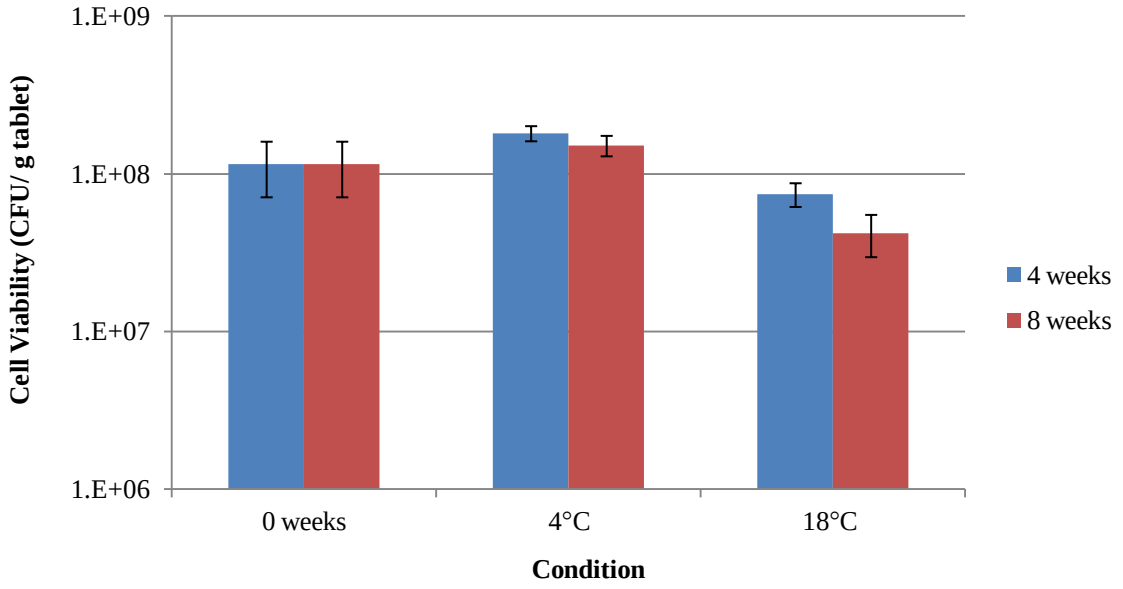


Figure 6. 7 Changes in cell viability of MG03 tablets (50 %(w/w) yeast: 50 %(w/w) powder blend, Formulation 7) produced at compaction force of 14 kN (pressure ca. 105 MPa) and speed of 30 mm min^{-1}, stored in cold and room temperature conditions, for 4 and 8 weeks. Mean ± 95% CI (n=3)

A general trend of decrease in cell viability was seen with tablets stored in laboratory conditions (18°C and 40 +/- 10% RH), 0.2 log loss after 4 weeks and 0.4 log loss after 8 weeks. It seems strong correlations cannot be seen with such a small scale study, therefore, conclusions cannot be drawn reliably. A long term storage study was initially planned to be conducted at Lesaffre Human Care and Feed Additives (France) however, the test was not carried out due to business reasons thus results were not be available.

6.2.5.1 Change in cell viability of Milled 3441 granules over time

A bulk sample of dried yeast Milled 3441 granules provided by Lesaffre Human Care and Feed Additives (France) (prepared as described in Chapter 3, Table 3.1) were stored under vacuum at 4°C, from which small samples were taken at time intervals and tested in triplicates for their viability (refer to Chapter 3, Section 3.2.2 for the cell enumeration method). The results would provide an indication for the storage stability of Milled 3441 granules. As shown in Figure 6.8, no change in cell viability was detected, indicating the long lasting stability of Milled 3441 granules. The large errors must be considered, which may be due to environmental changes in the laboratory or other uncontrolled experimental factors. An alternative explanation could be that there is a large variability in how individual granules react to storage conditions, considering the wide particle size range and different granule composition.

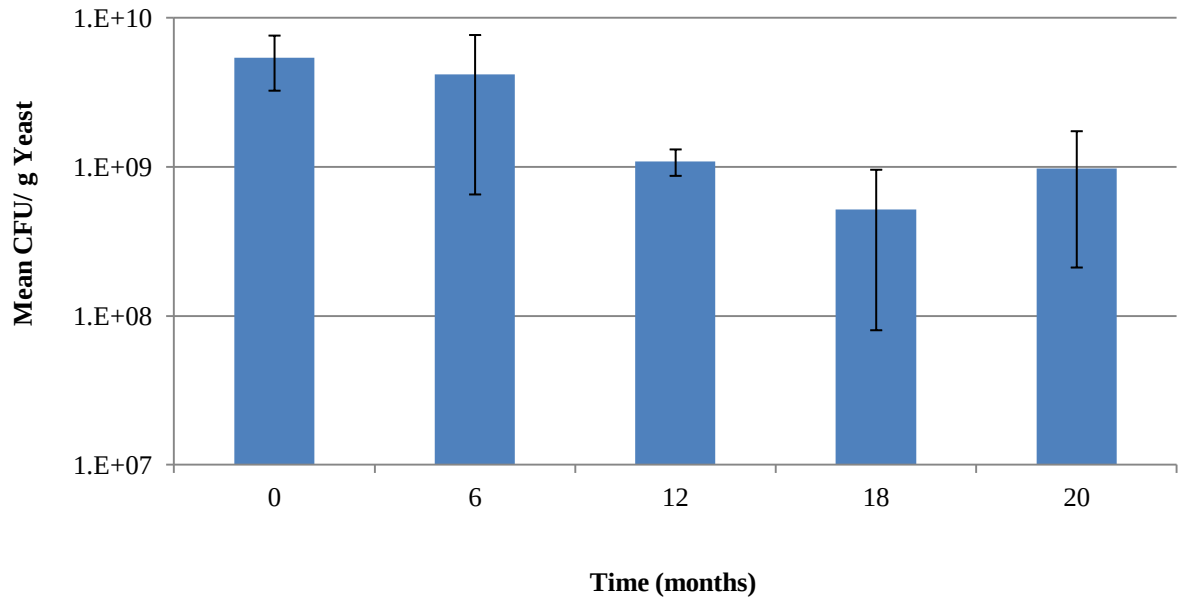


Figure 6. 8 Cell viability of samples taken from a bulk of Milled 3441 yeast granules stored in a 4°C fridge and tested over time. Mean ± 95% CI (n=3)

6.2.6 Reproducibility test

The term "uniformity of dosage unit" is defined as the degree of uniformity in the amount of the drug substance among dosage units (Fang et al. 2009). The lead formulation (50:50%(wt.) yeast: powder blend) of MG03 was tested further for reproducibility of viable yeast content and uniformity (homogeneity). The latter was important, as a difference in particle size was seen between MG03 granules and powder particles (refer to Chapter 4, Section 4.2.1.1) which might have caused segregation during tabletting, resulting in differences in the cell dosage per tablet. The cell viability of 5 MG03 tablets selected from a batch of 20 was determined (methods outlined in Chapter 3, Section 3.2.4.1). As shown in Figure 6.9, very little variability of cell viability was seen, taking into account the errors. In this case, the mean was calculated from triplicate agar plates.

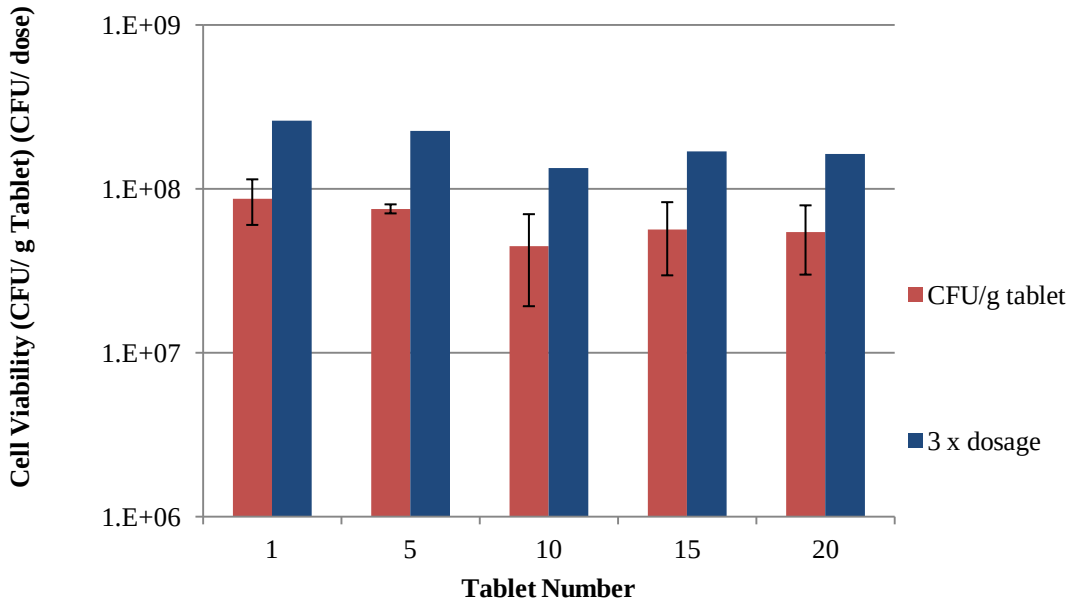


Figure 6. 9 Yeast viability uniformity test for probiotic tablets containing MG03 (50 %(w/w) yeast: 50 %(w/w) powder blend, Formulation 7), produced at a compaction force of 14 kN (pressure ca.. 105 MPa) and speed of 30 mm min^{-1}. Mean + 95% CI (n=3)

The cell viability of MG03 samples and tablets produced from the same sample batch was investigated over three months, to determine if storage of dried yeast had an effect and to confirm the dosage of tablets produced at different time points. Reproducibility in cell viability was found for MG03 samples and tablets from August to October 2013 (Figure 6.10). Results were also found to be consistent with those received from Lesaffre Human Care and Feed Additives (France), with no significant difference (p<0.05) in cell viability of the control MG 03 sample. This indicates the reproducibility of tablet dosage form.

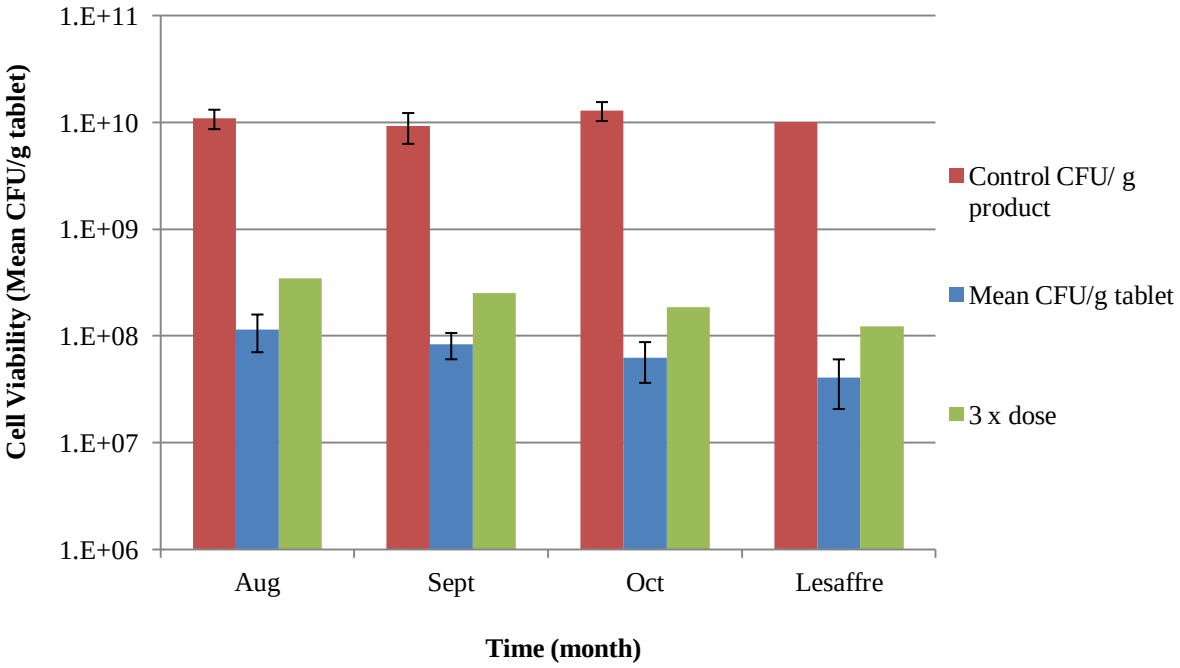


Figure 6. 10 Cell viability of MG03 tablets produced at various time points over 3 months to determine the reproducibility of the lead formulation. Mean ± 95% CI (n=3)

6.3 CONCLUSION

New samples comprising of various coating materials and compositions were received (Table 6.1). Based on the preliminary results (of tablet strength and cell viability), Wax M (non- agglomerated), Wax P and MG03 were selected as lead formulations, in addition to Milled 3441 outlined in Table 6.2. Upon the selection of a tablet formulation, it is important to test the final product further for various properties concerning functionality and stability. The dissolution rate, effect of temperature and storage time, and reproducibility of dosage per tablet are some factors that need to be considered and tested in determining the suitability of the product for consumers.

A friability test was conducted using these tablets of lead formulations. The results were used to determine if any trends were present with respect to tensile strength. However, a weak correlation (R^2= 0.59) was found between an increasing tablet tensile strength and reduction in friability. Using the diametric compression test to determine the tensile strength, as a way of characterising tablet strength, needs to be considered since these techniques are relied on during formulation development of tablets.

For tablets containing Milled 3441 yeast granules in a ratio of 50:50 %(w/w) yeast: powder blend, a plateau of cell viability release was seen at 40 min for pH 7 dissolution medium and 80 min for pH 2. No significant difference (p<0.05) was seen in the total cell viability between the two media indicating low pH did not have a detrimental effect on yeast cells.

A general trend of decrease in cell viability was seen with MG03 tablets stored at room temperature conditions; 0.2 log loss after 4 weeks and 0.4 log loss after 8 weeks. Upon receiving data from a long term test being conducted at Lesaffre International, conclusions might eventually be drawn regarding the effect of temperature and humidity on tablets containing MG03 dried yeast.

Tablets comprising of 50:50 %(w/w) MG03 yeast: powder blend was selected as the lead formulation for probiotic tablets containing dried yeast. These probiotic tablets exhibited a tensile strength of 1.4 ± 0.1 MPa and contained 1.15 $x10^8$ ± 4.43 $x10^7$ CFU/ g tablet, resulting in a daily dose of 3.5 $x10^8$ CFU /g in 3 doses (tablets), determined using the methods outlined in Chapter 3 Section 3.2.4. Reproducibility of tablets with respect to cell viability was found across numerous batches and after production over 3 months. A formulation for the production of probiotic tablets containing dried yeast has been identified for patient and consumer use.

CHAPTER 7: FINAL CONCLUSIONS AND RECOMMENDATIONS

7.1 OVERALL CONCLUSIONS

A formulation to produce dried yeast probiotic tablets has been developed because of the advantages of tablet dosage forms and their popularity. Yeast granule samples received from Lesaffre Human Care and Feed Additives (France) were screened based on their viable cell number per gram and compaction properties from which Actisaf STD and Pro GI Milled 3441 were bought forward for further work, including particle characterisation and formulation optimisation.

The particle size of yeast granules was found to be significantly larger (Actisaf STD the largest, diameter = 1252 ± 113 µm) than the particle size of MCC and DCP powder excipients (DCP the smaller, diameter = 22 ± 2 µm), resulting in segregation during mixing and tabletting. The mechanical properties of single powder particles and yeast granules were determined using a micromanipulation technique and a Zwick/Roell Z030 mechanical tester, respectively, concluding that the mean stress required to cause rupture was highest for Actisaf STD granules (15 ± 0.2 MPa). Tablets of various compositions including single and binary mixtures were produced (at a compaction pressure of 105 MPa) and tested using a diametric compression test, highlighting the excellent binding properties of MCC (tablets having a mean tensile strength of 5.5 ± 0.1 MPa). DCP displayed poor binding properties when compressed alone (mean tensile strength of 0.47 ± 0.09 MPa) as a result of its brittle fracture deformation mechanism (Klevan et al. 2010, Rowe et al. 2009), but the mean tablet tensile strength improved to 2.85 ± 0.04 MPa, when combined with MCC, in agreement with Jivraj et al. (2000). The force required to cause rupture of particles was then used to predict the

compaction behaviour using the Kawakita model. Single MCC particles did not rupture under diametric compression and compaction of Actisaf STD granules did not form tablets that could withstand handling, therefore, correlations were sought with results obtained from Milled 3441 yeast granules and DCP particles. It was found that the brittle behaviour of DCP primary particles, when compacted alone, resulted in tablets with lower tensile strength values (0.47 ± 0.09 MPa) than those tablets produced of MCC particles behaving plastically (5.5 ± 0.1 MPa).

The relationship between 1/*b* (derived from the Kawakita model equation) and the nominal rupture stress of single particles was sought, but due to the lack of sufficient data no conclusion could be drawn. In addition, a correlation between the tensile strength of single component tablets and their corresponding 1/*b* values was found. Primary particles with high 1/*b* values resulted in tablets exhibiting a low tensile strength. The strong properties of primary particles, not rupturing easily, exhibited less surface area available for bonding, thus producing weak tablets. This is a novel approach since there is little literature available. Testing the compression of a wider range of primary particles (including yeast granules and powder excipients), more data points would be obtained allowing stronger trends to be determined.

When investigating the effects of formulation on viable cell number post-tabletting, it was found that tablets of DCP-Milled 3441 yeast resulted in a higher viable cell number ($1.2 \times 10^8 \pm 0.3 \times 10^8$ CFU /g tablet), than tablets of MCC-Milled 3441 yeast ($8.5 \times 10^7 \pm 1.5 \times 10^7$ CFU /g tablet). This may be due to the difference in fragmentation behaviour of powder particles during compression, DCP exhibiting brittle fracture (causing less damage to granules)

compared to the plastic properties of MCC. Once again, this highlights the impact that the behaviour of primary particles has, on cell viability after compaction.

The formulation of probiotic tablets was developed further using the dried Milled 3441 yeast granules by varying the ratio of yeast granules: powder blend, based on a selected powder blend formulation consisting of 25% MCC and 24.25 % DCP, with 0.75 %(w/w) Mg St as the lubricant. This formulation favoured over others of similar tablet properties due to the inclusion of DCP, which is considered as a source of calcium in nutritional supplements (Rowe et al. 2009). The optimisation results indicated Formulation 12, containing the ratio of 75 %(w/w) yeast granules: 25 %(w/w) powder blend was best, based on the tablets exhibiting a reasonably good mean tensile strength of 0.9 ± 0.02 MPa and an adequate mean cell number of $9.1x10^8 \pm 0.5 \times 10^8$ CFU /g tablet. During the testing of processing conditions, it was found that increasing compaction pressure from 105 to 150 MPa resulted in tablets with increasing tensile strength (MPa), but had an adverse effect on cell viability (which decreased significantly). Speed of compression, on the other hand, was seen to have a significantly adverse effect on tablet strength; a 35% decrease in mean tensile strength was observed with compaction speed increasing from 1 to 60 mm min^{-1}, with no effect on the cell viability. The reduction in tensile strength is explained by those particles undergoing time dependant compaction behaviour, mainly by plastic deformation (Tye et al. 2005) i.e. MCC particles.

Based on the formulation development of powder blend and yeast : powder blend variations, a protocol formulation was developed comprising of 50% Milled 3441 yeast , 25% MCC, 24.25% DCP and 0.75%(w/w) Mg St. This protocol formulation was then applied to new yeast samples of different coating materials, including variations of wax and lipid

composition (Chapter 6, Table 6.1). It was found that the formulation can be applied to a variety of yeast coated granules, which result in tablets meeting the specification of exhibiting a tensile strength of 1 MPa or cell viability of 2 x 109 CFU per 3 tablets. Tablets of MG03 resulted in a mean tensile strength of 1.4 ± 0.1 MPa, a friability weight loss <1%, and contained 1.2 $x10^8$ ± 4 $x10^7$ CFU/ g tablet, resulting in a daily dose of 3.5 $x10^8$ CFU in 3 doses (of 1 g tablets). This was not the value desired by Lesaffre, Human Care and Feed Additives (France) as 3 tablets do not achieve 2 x 10^9 CFU in 3 doses, however a formulation resulting in tablets considered strong enough to withstand production, handling and transportation has been developed. In order to improve the cell viability after compaction, whilst maintaining tablet strength, the mechanism of cell damage during compaction is essential, as well as which is discussed in the recommendations section below.

7.2 RECOMMENDATIONS FOR FURTHER WORK

Given the results of the research described here, a formulation of powder excipients and dried yeast as the active, has been developed for the application of probiotic nutritional supplements. In future work, other techniques to characterise the mechanism of cell damage during tabletting process, should be used to form greater understanding, which can lead to developments to further prevent such damage. A well established technique for the analysis of individual cell physiology includes fluorescent staining and flow cytometry which provides information regarding cell functions (i.e damage to cell memebrane). Using this technique with Propidium Iodine (PI) and Bis-oxonol (BOX), cells can also be defined as “healthy, injured and dead”. Such an approach has been taken by a fellow student in the University of Birmingham. By forming an understanding of the mechanism of damage to

yeast cells under a compaction load, methods of preventing this, or providing further protection could be achieved. This information could also be used to better understand the compaction behaviour of dried granules during tabletting to improve tablet strength.

Upon the selection of the final formulation, further testing would be advisable. A dissolution study, according to Ph. Eur. Standards, comprising of placing 6 tablets in pH 2, pH 6.8 and phosphate buffer saline would be recommended. This would provide an indication of the dissolution profile over time, and depending on the results, would determine if a disintegrant is required or not. However, the Ph. Eur. guidelines may not always be applicable for probiotics, such as for when the intention of tablets is for supplement or nutritional purposes (i.e non medicinal). Using the various buffers (outlined in the guidelines), would provide additional information with regards to the effect of a range of pH values on tablets containing dried yeast and its viability upon release since the mode of action of probiotics occurs in the small intestine where a high pH is present.

All the experimental work was conducted on a laboratory manual press, thus the prospect of validation at a pilot scale could be explored in future work. It was found that when compaction speed was increased to 60 mm min^{-1}, the tensile strength of tablets decreased. Since the speeds used on a commercial level are significantly higher, these results provide a predictive indication of the negative effects compaction speed has on tensile strength. Further studies on how scale up of compaction would affect tablet properties would need to be conducted, since the effects of speed on a laboratory single press can differ from that on a continuous process scale. As higher compaction speeds are employed during large scale production, the effects of scale- up on the mechanical strength properties of tablets would

also need to be considered, since problems such as low tensile strength and capping still exist in large- scale production (Celik 2011).

Large scale tubular mixers could be used, in addition to a rotary tablet press instead of a single press. During scale up, other factors such as the flowability of yeast and powder during the filling stage are also important and should be characterised. Avalanche testing is recommended as a method to obtain the Carr's Index and Hausner's ratio, which can be used to determine the compressibility index (Ph. Eur. 5.0, Chapter 2.9.36). This would also indicate the feasibility of filling the die via a hopper instead of manual filling. As a final step to tablet preparation, film coating could be conducted on tablets. This would provide numerous advantages including aesthetic appeal, masking the segregation of particles and improving the ability of the user to swallow the tablets (Porter 2013). Film coating can also increase the mechanical strength of tablets, implying that a lower compaction pressure is required, which can lead to tablets exhibiting higher cell viability. In this case, tablets with initial low tensile strength with high yeast viability could be produced with an additional coating step as a way to increase the final tablet strength.

Yeast probiotic tablets could be applied for additional intentions to animals use (Fuller 1989). Digestive supplements and oral care for pets, fish care products, and food supplements for commercial farm animals (e.g. poultry, pigs, horses, etc.) (Foligne et al. 2013) are becoming of high interest. The current formulation would need to modify to meet the criteria for cattle use, such as a higher dosage.

APPENDIX 1

Protocol for the enumeration of total living yeast cells provided by Lesaffre (France).

I. APPLICATION

This method is applicable to the yeast ufc (unites forming colony) enumeration.

II. PRINCIPLE

A medium, containing adequate nutrients for growth of most yeasts and moulds and antibiotics for inhibition of most bacteria, is inoculated with a given quantity of the product. It is incubated at 25°C for 3 to 5 days. Colonies appearing on the medium are then counted and/or examined.

III. PRECAUTIONS

Some yeasts and moulds can be infectious or can cause allergic responses; therefore, it is important to be cautious when working with fungi. Ideally, plates should be held in incubators, not in an open room. Plate lids should generally only be removed for things such as the preparation of a slide for microscopic examination. Flamed needles should be cooled before making transfers to avoid dispersal of conidia and other cells. Avoid inhalation of spores and other fungal products such as volatiles (do not smell).

IV. MATERIALS AND EQUIPMENT

The media uses in this method (3.2) are commercially available and are to be prepared and sterilized according to the manufacturer's instructions. See also Appendix 1 for the formula of individual media.

3.1 Material

- Sterile spreader
- Light microscope
- 1 ml sterile graduate transfer pipets (filled in with cotton)
- Tubes (20mL)
- ∅ 90 mm
- Magnetic stirplate
- 1 L flask
- Sterile flasks (100mL) with "magnetic bar"
- Incubator capable of maintaining 25°C
- Water baths at 100°C and 48°C (to temper agar)
- Precision weighing balance ± 0.001g

NOTE: It is the responsibility of each laboratory to ensure that the temperatures of the incubators or water baths are maintained at the recommended temperatures. Where 35°C is recommended, the incubator may be 35 +/- 1°C. Similarly, lower temperatures of 30 or 25°C may be +/- 1°C. However, where higher temperatures are recommended, such as 43 or 45.5°C, it is imperative that the incubators or water baths be maintained within 0.5°C due to potential lethality of higher temperatures on the microorganism being isolated.

3.2 Reactive agents and culture medium

- TS buffer solution (Tryptone salt) (see. group method MIC003)
- YM agar media (see appendix 1)
- Oxytetracycline solution (1% solution)

V. PROCEDURE

5.1 Preparation for analysis

For the standard practices, refer to the group method MIC002 derived from the NF ISO 7218 standard.

Clean surface of working area with a suitable disinfectant.

Mark clearly the Petri plates identifying sample, sample unit, dilution or date of inoculation as required for identification.

Prepare the appropriate media for the analysis being carried out. Before use, the media is maintained to 48°C. For 100 mL of agar medium, add 1 mL of oxytetracyline solution (1%). Ensure pre-poured plates are dry before use, by allowing plates to dry at room temperature overnight, or other suitable means (e.g., laminar flow hood until dry).

5.2 Preparation and dilutions of the sample

Weigh with accuracy 1 g of sample in a sterile flask.
Add 100 mL of TS buffer solution pre-warmed to 37°C.

The suspension is homogenized using an UltraTurax homogenizer (10000 to 12000 rpm for 3 min)

Homogenize the suspension before using it and prepare succeeding decimal dilutions as required (in distilled water pre-warmed to 37°C), using a separate sterile pipette for making each transfer

5.3 Plating and incubation

Yeast that may have been stressed should be enumerated by a surface spread plate technique rather than with pour plates. This technique provides maximal exposure of the cells to oxygen and avoids heat stress from molten agar. Pour plates may be used for all yeasts at the lab's discretion and if validated that the counts are not significantly different between spread plates and pour plates (Beuchat, L.R., Nail, B.V., Brackett, R.E., Fox, T.L.. "Comparison of Pour and Spread Plating for Enumeration of Fungi in Foods." 08/20/1990. 31: 27 - 30, 1992).

Transfer to the surface of the plate (with 1mL sterile pipet), 0.1mL or 0.5mL of the required dilutions to appropriate Petri plates. Gently spread the liquid culture onto the surface of the agar by moving the spreader in a circular manner while rotating the plate. This will ensure an even distribution of yeast.

Incubate plates in a reversed position to 25°C for 3 days.

We propose for example to spread 0,1mL of 10^{-8} dilution on 3 x ∅ 90 mm Petri dish, spread 0,1mL of 10^{-7} dilution on 3 x ∅ 90 mm Petri dish and finally spread 0.1mL of 10^{-6} dilution on 3 x ∅ 90 mm Petri dish.

Yeast Malt Extract Broth (Formulation per Liter g/Liter)

- Glucose10.0	10
- Peptone 5.0	5
- Yeast Extract 3.0	3
- Malt Extract 3.0	3
- Agar	20

pH 6.2+/-0.2

Oxytetracycline 1%

- Oxytetracycline	1g
- Sterile demineralised water	100g
- HCl	2mL

Keep the solution at 4°C in a dark room

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