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Stability of the probiotic *Lactobacillus paracasei* CRL 431 under different environmental conditions

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Devastotra Poddar

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Abstract

Probiotics are live microorganisms which provide health benefits to the host upon consumption. There is a wealth of information available on the health benefits associated with the consumption of probiotics. However, currently probiotic microorganisms are delivered mainly through refrigerated, short shelf-life products. When incorporated into ambient shelf-life products, the products generally fail to meet the regulatory criteria, which require probiotic bacteria to be viable in high numbers at the end of shelf-life. Storage temperature, oxygen and residual moisture content often result in loss of viability of probiotics during storage and distribution.

A preliminary study was carried out to explore the effects of matrix composition (fat, protein and carbohydrate) on the probiotic bacterial (*Lactobacillus paracasei* CRL 431) viability, during fluidized bed drying and subsequent storage. The finding suggests that whole milk powder provided a superior protection to bacteria during fluidized bed drying and subsequent storage, compared to skim milk powder or milk protein isolate. Moreover, water activity of the powders during storage played a key role in determining the probiotic viability.

The effects of drying techniques, moisture content and water activity on the storage stability of *L. paracasei* in a whole milk matrix were studied. Whole milk powder-bacteria mixtures were dried using spray drying, freeze drying or fluidized bed drying and stored at 25 ºC under controlled water activity (0.11 a_w, 0.33 a_w and 0.52 a_w) for 105 days. At 0.11 a_w, cell viability loss was minimal, while at 0.52 a_w viability was lost in all powders within 22 days. At the intermediate 0.33 a_w, there were marked differences among stored powders. Further, various analytical techniques (X-ray diffraction, FT-IR, Raman, NMR spectroscopy) were used to explore why and how structural differences in the matrix-bacteria mixtures, produced using different drying technologies, under different water activity storage conditions, influence bacterial viability. The results suggest that fluidized bed drying provided a better protection to the bacteria during storage, which was attributed to unique powder structure that reduced the absorption of water. The lower absorption of water resulted in the maintenance of a more rigid structure, which limited molecular mobility.

Lactobacillus sp. is known to accumulate large amounts of inorganic manganese which apparently provides defense against oxidative damage by scavenging free radicals. The

ability of *L. paracasei* to maintain viability during long term ambient storage may be enhanced by the ability of microorganism to accumulate manganese, which may act as free radical scavenger. To investigate this hypothesis, X-ray fluorescent microscopy (XFM) was employed to determine the changes in the elemental composition of *L. paracasei* during growth in MRS medium with or without manganese as a function of physiological growth state (early log vs. stationary phase). The results revealed that lower level of manganese accumulation occurred during the early log phase of bacterial growth compared with the stationary phase cells. The lower level of manganese accumulation was found to be related to the loss in bacterial viability during storage.

Manganese has been known to possess pro- and anti-oxidant properties, and understanding of the changes in the manganese oxidation state was considered to provide some further insights into the bacterial death mechanisms. In view of the relatively high concentration of manganese in lactobacilli, it was of interest to better understand the oxidation state, coordination number and ligands of the manganese in the bacteria. It was possible to characterize the changes of manganese within bacteria using XANES. The results confirmed that manganese present within *L. paracasei* is in Mn(II) oxidation state and no changes in the manganese ligands could be observed during storage.

In summary, the thesis provides a mechanistic insight into the ways to improve the stability of probiotics for application into ambient long shelf-life products. Future studies on tracking the genetic and proteomic aspects of the bacteria during storage might be useful for further understanding and process optimization.

Keywords: Probiotics, freeze drying, spray drying, fluidized bed drying, FT-IR, NMR.

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Abbreviations

a_w	Water activity
AO	Acridine orange
CLSM	Confocal laser scanning microscopy
DSC	Differential scanning calorimetry
EDS	Energy-dispersive X-ray spectroscopy
EELS	Electron energy loss spectroscopy
ESR	Electron spin resonance spectroscopy
EXAFS	Extended X-Ray Absorption Fine Structure
FT-IR	Fourier transform infrared spectroscopy
GC	Guanidine cytosine
MPI	Milk protein isolate
PI	Propidium iodide
SEM	Scanning electron microscopy
SMP	Skim milk powder
TEM	Transmission electron microscopy
TGA	Thermo gravimetric analysis
WMP	Whole milk powder
XANES	X-ray absorption near edge structure
XAS	X-ray absorption spectroscopy
XFM	X-ray fluorescence microscopy
XRD	X-ray diffraction

Chapter 1 General Introduction

1.1 Introduction

Today healthcare cost is at an all-time high, unsustainable and continues to rise (ANPHA, 2013). Governments and organizations around the world are turning to work-place health programs, to help employees adopt healthier lifestyles to lower the risk of chronic diseases. In pursuit of good health, people have understood the importance of food in causing and preventing diseases. They are looking to adopt the viewpoint of Hippocrates “let food be thy medicine and medicine be thy food”, and adapt more healthy practices. As a result of change in consumer mindset, there has been increased demand towards food which improves their overall health. Over the years, food and beverage industry has developed functional foods that eliminate nutritional deficiencies, improve gut health and immunity. There are dozens of successful examples of fortification compounds, such as vitamins, minerals and probiotics added commercially in food products to enhance health benefit. During the last decade, there have been more than 8,000 research articles indexed in PubMed containing the term “probiotic”, and establishing its effectiveness in controlling chronic diseases (Hill et al. 2014).

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). The market for probiotic foods was projected to reach US$20 billion by 2020 (Siro et al. 2008) and deserved to be recognized as a top functional ingredient. Offering the consumer with diverse range of probiotic food products, some, manufacturers led to misuse of the term “probiotic”, without meeting the required criterion. With a view to re-examine the concept of probiotics, and to protect the consumers from misleading health claims, International Scientific Association for Probiotics and Prebiotics (ISAPP) meeting was held on 23rd

October 2013, bringing together clinical and scientific experts. The Consensus panel outcome was to keep live cultures, traditionally associated with fermented foods, for which there is no evidence of a health benefit, outside the probiotic framework. Probiotic framework must only include microbial species that have been shown to provide health beneficial effect in well conducted controlled human studies, and delivery of viable probiotics at efficacious dose at the end of shelf life (Hill et al. 2014).

Many of the probiotic products available in the market would largely fail the end of shelf life test, if they had to meet viable probiotic count so as to claim for probiotic benefit. Probiotic organisms have therefore only found widespread application into fresh foods, such as yoghurt and fermented milks, which have refrigerated shelf life of few weeks. However, today's consumers need healthy food in various formats (Chavez and Ledeboer, 2007). For large-scale adoption, probiotics need to be delivered via ambient, long shelf-life food products. However, it is a challenge for the food researchers to develop ways of protecting probiotic bacteria in an ambient shelf-life product. Storage temperature, oxygen and residual moisture content often result in loss of viability of probiotics during storage in dried products. In order to improve their viability during storage, probiotic bacteria must be either embedded in a protective matrix or undergo cell physiology maneuver or a combination of both, before incorporation in the long shelf-life dry products (Corcoran et al. 2004; Ananta et al. 2005).

Researchers have attempted a flurry of techniques to improve the viability of probiotic bacteria during storage. These technologies include:

1. Stabilization using drying or desiccation, such as freeze drying, spray drying, fluidized bed drying and vacuum drying or mixed/ two-step drying process (Ananta et al. 2005; Simpson et al. 2005; Santivarangkna et al. 2007).

2. Encapsulation processes, such as emulsion techniques, extrusion techniques and coacervation (Hsiao et al. 2004; Mokarram et al. 2009).

3. Stabilization by manipulating the cell physiology, stress adaptation by heat stress, osmotic stress, acid stress, optimization of growth phase, cell harvesting conditions, growth media and growth conditions (Van de Guchte et al. 2002; Prasad et al. 2003; Corcoran et al. 2004).

4. Optimization of storage and packaging conditions.

To date, only drying/desiccation techniques have gained popularity because of their commercial availability (Hsiao et al. 2004). All the drying processes, used to embed bacteria result in some loss of viability. In freeze drying, this is due to ice crystal formation and rupture of the cell membrane (Fonseca et al. 2004). In spray drying, the high temperature results in the thermal inactivation of the cells (Ananta et al. 2005; Fonseca et al; 2000, Teixeira et al; 1995). In contrast, fluidized bed drying uses mild temperatures and therefore minimizes bacterial inactivation (Bayrock and Ingledew, 1997). Fluidized bed drying has been widely used in the production of wine yeast, but has found limited application in the production of concentrated viable bacteria (Caron, 1995). However, a recent study showed that fluidized bed drying of the bacterial cells in a dairy matrix improved the stability of probiotic *Lactobacillus* sp., with a viable cell count of 100 million cells per gram of dried probiotic powder after 52 weeks of storage at 25˚C (Nag and Das, 2011).

During the drying processes, the matrix composition (carbohydrates, fat and protein) and structure protects the bacteria against desiccation or storage stress, thereby maintaining bacterial viability. Carbohydrates, such as lactose used in the drying matrix, substitute the hydrogen bonded water in the head group region of the phospholipid bilayers

present in the bacterial cell membrane (Leslie et al. 1995, Morgan et al. 2005, Santivarangkna et al. 2008). In addition, the amorphous (glassy) state of the carbohydrates, during the drying process, imparts very high viscosity, which can act as a protective encapsulation for the bacteria, limiting water and oxygen exchange (Morgan et al. 2006). Proteins can delay the crystallization of carbohydrates to maintain the protective glassy state (Haque and Ross, 2004; Adhikari et al. 2007). Similarly, fat may also delay the crystallization of lactose (Kim et al. 2003; Knudsen et al. 2002). Bacterial viability during storage depends on the moisture content, which in turn, is influenced by the water activity conditions during storage (Gunning et al. 1995; Hsiao et al. 2004; Miao et al. 2008). In order to maintain viability for long periods, the carbohydrate must remain in glassy (non –crystalline) state, for example, 0.11 a_w. At higher water activity, water begins to act as a plasticizer of the protective carbohydrate glass and increases the molecular mobility. The increase in molecular mobility results in crystallization, with instantaneous loss of bacterial viability during storage (Miao et al. 2008).

1.2 Outline of the thesis

The way problems are conceived has a massive impact on the technological approach used to solve them. Neither the protective mechanism of the drying matrix nor the drying process of probiotics is as yet fully understood (Chapter-2, literature review). Therefore, a preliminary study (Chapter-3) was carried out to link the use of matrix constituent, i.e. fat, protein and carbohydrate (lactose), with the decline in probiotic bacterial (*Lactobacillus paracasei* 431) viability, during fluidized bed drying and storage. The finding suggests that whole milk powder (containing predefined mixture of fat, protein and lactose), may provide superior protection to bacteria during fluidized bed drying and storage, compared to skim milk powder (protein and lactose) or milk protein isolate

(protein). Moreover, water activity of the powders during storage played a key role in determining probiotic viability counts. The study provided additional information and identified the drying matrix and storage water activity selection for future experiments (Chapter 4). Chapter 4 investigates the effects of drying technique (freeze drying, spray drying, fluidized bed drying), moisture content and water activity on the storage stability of *L. paracasei* in a whole milk matrix. Further, the experiments explore why and how structural differences in the matrix-bacteria mixtures, produced using different drying technologies, under different water activity storage conditions, influence bacterial viability. The results suggest that fluidized bed drying provided better protection to the bacteria during storage, which was attributed to unique powder structure that reduced the absorbtion of water. The lower absorption of water resulted in the maintenance of a more rigid structure, which may limit molecular transport, particularly of oxygen.

L. plantarum is known to accumulate large amount of manganese (Mn), which provides protection to the bacteria against oxidative damage. The ability of *L. paracasei* to maintain viability during long term ambient storage may be enhanced by the ability of microorganism to accumulate manganese, which may act as free radical scavenger. To investigate this hypothesis, Chapter 5 explores the levels of manganese accumulation in *L. paracasei* during early log-phase, stationary phase and bacteria grown in manganese deficient medium. The results of this study indicated that higher bacterial death was observed during storage, when the manganese accumulation was lower (early log phase cells and bacteria grown in manganese deficient MRS medium). Moreover, the drying matrix, used for immobilization, was observed to be deficient in manganese and hence manganese could be used as a marker to visualize the embedded bacteria.

It was, therefore, of interest to study the changes associated with manganese during storage at different water activity conditions, (Chapter 6). Electron spin resonance spectroscopy was used to identify and measure Mn (II) species in intact *L.paracasei cells.* Mn spectrum of the bacteria was observed to have close resemblance with that of manganese phosphate. ESR spectroscopy used in the present study was unable to detect whether there was Mn (III) formation during storage, resulting in increased bacterial death. Moreover, there was an ambiguity over the state and form of manganese in bacterial cells during storage. X-ray absorption spectroscopic analysis was carried out to understand *in-situ* speciation of manganese and to look for any change in Mn oxidation state during storage. No difference could be found in the chemical state of Mn between the embedded bacteria stored under different storage water activity conditions. The results also confirm the findings in Chapter 4 which suggest fluidized bed dried powder provided rigid structure compared to spray and free dried powders which might play a protective role by restricting molecular mobility. The storage conditions used in the study and the changes to bacterial viability of probiotic encapsulates do not alter the oxidation state and speciation of Mn in a way that might be harmful for human consumption.

The findings from this thesis suggest that using fluidized bed drying, it may be possible to produce dried probiotics in a milk powder matrix that have adequate storage life to provide health benefits to consumers. Further investigation and optimization of this technology may lead to commercial products and it may be possible to extend this technology to a broad range of probiotics.

Chapter 2 Literature Review

2.1 Background

This literature review covers the definition of probiotics, their health promoting attributes. It explores the various stabilization strategies that pose promising potential for incorporations of probiotics into food products. Finally, the literature review identifies possible gaps in our knowledge and highlights the need for further mechanistic understanding of how probiotics could be delivered in ambient shelf-stable products.

2.1.1 Definition of probiotics

The widely accepted definition of probiotic was envisaged by Fuller (1992), in which he stresses the significance of live cells as a requisite for an effective probiotic. Probiotics were defined as living microorganisms which when ingested have beneficial effects on the host by improving the physiological functions of the intestinal microflora (Fuller, 1992). This definition was broadened by Havenaar and Huis In't Veld (1992), in which he refers probiotics to a "mono-or mixed culture of live microorganism which beneficially influences the man or animal by improving the properties of the indigenous microflora". Tannock et al. (2000) later defined probiotics as "live microbes which transit the gastro-intestinal tract and in doing so benefit the health of the consumer". Expert consultation of international scientist working on behalf of Food and Agricultural Organization of the United Nations and the World Health Organization reworked the probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Since then, it has become widely adopted version of probiotic definition worldwide. With an increase the scientific evidence of health benefits of probiotics, manufactures launched several probiotic products

in market. However, the misuse of the term “probiotic” has become a major issue before food regulatory authorities. In order to reexamine the concept of probiotics and to remove inconsistencies between the FAO/WHO report and guidelines, International Scientific Association for Probiotics and Prebiotics (ISAAP) meeting was held on 23rd October 2013. Expert panel recommended, for probiotic in food or supplement with a specific health claim, should contain (1) defined probiotic strain(s) and have (2) proof of delivery of viable strain(s) at efficacious dose at end of shelf-life (Hill et al. 2014).

2.1.2 Probiotics and health benefits

The health benefits associated with probiotics have been studied extensively over the past century. In early 1900, the Russian Nobel Prize winner, Elie Metchnikoff, observed an increased life expectancy in Bulgarians who consumed fermented milk products. Later, he advanced the theory that the consumption of lactic acid bacteria would minimize intestinal putrefaction and prolong life (Metchnikoff, 1907). Overall, cultured milk has become an integral part of diets because of the health-promoting attributes associated with its consumption. There are several possible modes of action by which probiotic bacteria provide health benefits to the host (Gareau et al. 2010) (Figure 2.1). These include suppression of pathogenic bacteria by producing antimicrobial compounds and competing for adhesion sites and nutrients and alteration of the microbial metabolism by controlling enzymatic activity in the intestine (Fuller, 1991). Further, probiotics stimulate immunity by increasing T cell production (Anukam et al. 2008; Baron 2009) and modulating cytokines, tumor necrosis factor-α, interleukins, and interferons (Borruel et al. 2002; Bai et al. 2006; Chen et al.2009a). Moreover, they inhibit the epidermal growth factor receptor and insulin-like growth factors in tumor cells (Chen et al. 2009b). Finally, increased intestinal antibody and macrophage levels enhance the phag-

ocytic capacity of the blood leucocytes (Gill et al. 2001). Thus, probiotics enhance protection against diseases.

Good probiotics should confer health benefits to the host animal. They should also be nontoxic and nonpathogenic in nature. Further, they should be capable of surviving at low pH and in the presence of the high concentration of bile salts in the gastrointestinal system (Fuller, 1991). In addition, they should be stable under storage conditions and should be easy to handle for industrial production. Overall, possession of the above properties will make the bacteria suitable for food and pharmaceutical applications.

From the 1990s to the present day, there has been a steady accumulation of scientific evidence that emphasizes the health benefits, including the control of chronic diseases, associated with the consumption of probiotics. Importantly, numerous experimental studies have emphasized the potential protective effects of *Lactobacillus* and *Bifidobacterium* species (Thantsha et al. 2009; Kumar et al. 2010) and their health benefits are summarized in Table 2-1

2.1.3 Applications of probiotics and their market potential

Probiotic bacteria are one of the top functional ingredients and underpin some of the most successful functional food brands in the world. The use of probiotics is limited mainly to foods that are subjected to chilled distribution and storage because there are currently few credible delivery technologies that are able to deliver probiotic bacteria in shelf-stable foods. This establishes ‘delivery technology for probiotic bacteria in shelf-stable foods’ as a significant opportunity of commercial importance because it will expand the current market for probiotics, which is estimated to be approximately US$20 billion by 2020 (Siró et al. 2008). Scientists generally agree and FAO/WHO guidelines state that we need to consume between 100 million and 1 billion live probiotic cells per

day to realize their health benefits. However, as a significant proportion of the bacterial cells are destroyed during processing, storage, and gastrointestinal transit, ‘stabilization of probiotic bacteria’ is an area of interest for research and development.

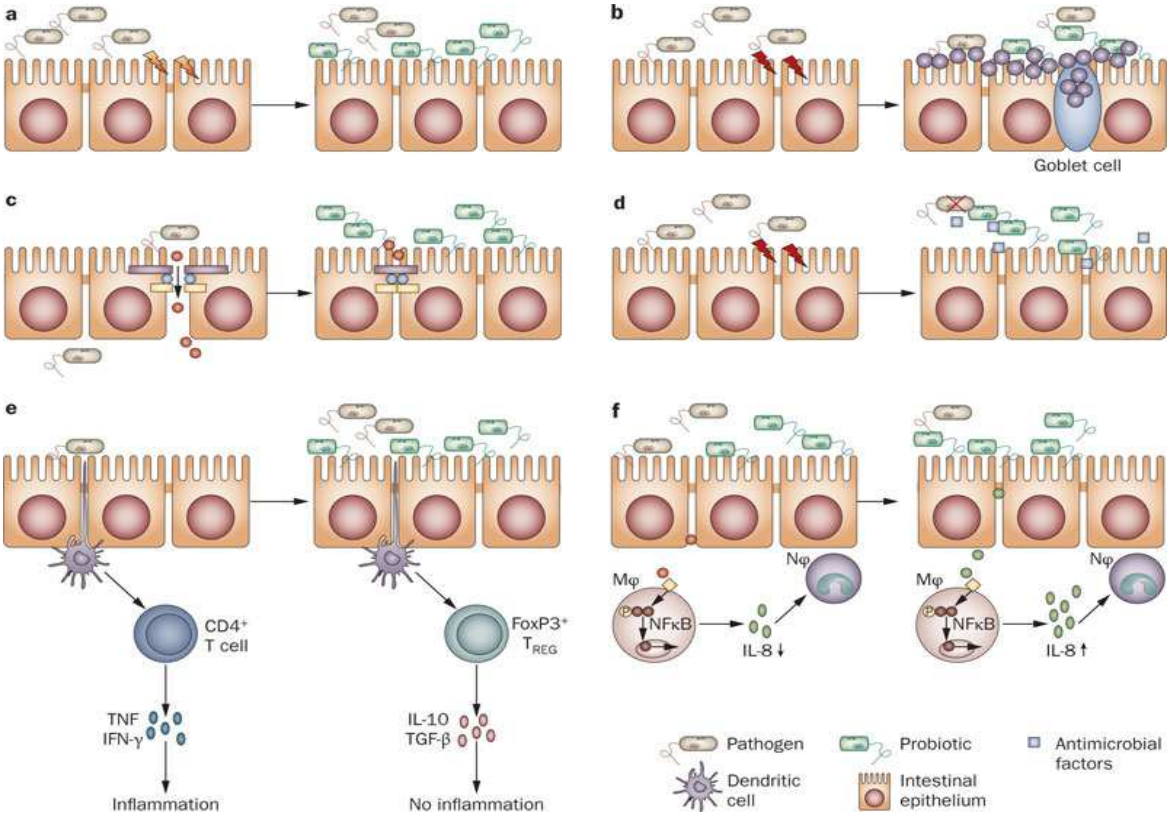

Figure 2-1 Potential mechanisms of actions of probiotics (Gareau et al. 2010)

Table 2-1 Selected studies showing the effectiveness of various strains of probiotic microorganisms against various diseases

Probiotic bacteria	Alleviation of disease symptoms	References
Bifidobacterium animalis	Irritable bowel syndrome, Dental caries, Rotavirus diarrhea, Fever, Rhinorrhea, Colitis	(Phuapradit et al. 1999; Çaglar et al. 2005; Rautava et al. 2006; Wildt et al. 2006; Guyonnet et al. 2007; Leyer et al. 2009)
Bifidobacterium bifidus	Necrotizing enterocolitis	(Bin-Nun et al. 2005)
Bifidobacterium breve	Irritable bowel syndrome, Respiratory infection, Refractory enterocolitis, Atopic dermatitis	(Kanamori et al. 2004; Saggioro 2004; Kukkonen et al. 2008; Yoshida et al. 2010)
Bifidobacterium infantis	Irritable bowel syndrome, Necrotizing enterocolitis, Diarrhea	(Hoyos 1999; Corrêa et al. 2005; Lin et al. 2005; O'Mahony et al. 2005; Whorwell et al. 2006)
Bifidobacterium lactis	Constipation	(Puccio et al. 2007)

Bifidobacterium longum	Ulcerative colitis, Respiratory tract infection, Common cold	(de Vrese et al. 2005; Furrie et al. 2005; Puccio et al. 2007)
Lactobacillus acidophilus	Necrotizing enterocolitis, Irritable bowel syndrome, Bacterial vaginosis, *Helicobacter pylori* therapy, Glucose intolerance, Hyperglycemia, Hyperinsulinemia, Dyslipidemia, Cancer, Fever, Rhinorrhea	(Hoyos 1999; McLean and Rosenstein 2000; Gaón et al. 2002; Xiao et al. 2003; Johnson-Henry et al. 2004; Saggioro 2004; Lin et al. 2005; Yadav et al. 2007; Leyer et al. 2009; Soltan Dallal et al. 2010)
Lactobacillus bulgaricus	Crohn's disease, Inflammatory bowel disease, Common cold	(Borruel et al. 2002; Şengül et al. 2006; Makino et al. 2010)
Lactobacillus casei	Crohn's disease, Constipation, *H. pylori* therapy, Arthritis, Glucose intolerance, Hyperglycemia, Hyperinsulinemia, Dyslipidemia, Diarrhea, Atopic eczema	(Kato et al. 1998; Tuomola et al. 1999; Gaón et al. 2003; Koebnick et al. 2003; Tursi et al. 2004; Kanazawa et al. 2005; Sýkora et al. 2005; Bu et al. 2007; Yadav et al. 2007; Cukrowska et al. 2010)
Lactobacillus fermentum	Bacterial vaginosis, Inflammatory bowel disease, Colitis	(Peran et al. 2006, 2007; Irvine et al. 2010)

Lactobacillus gasseri	Common cold, *H. pylori* therapy, Asthma, Allergic rhinitis	(Ushiyama et al. 2003; de Vrese et al. 2005; Chen et al. 2010)
Lactobacillus johnsonii	*H. pylori* therapy, Cirrhosis, Atopic dermatitis	(Cruchet et al. 2003; Inoue et al. 2007; Gotteland et al. 2008; Tanaka et al. 2008)
Lactobacillus paracasei	Allergic rhinitis, Irritable bowel syndrome, Diarrhea, Atopic eczema, Urogenital infection	(Verdú et al. 2004; Wang et al. 2004; Sarker et al. 2005; Zárate et al. 2007; Cukrowska et al. 2008)
Lactobacillus plantarum	Irritable bowel syndrome, Colitis	(Nobaek et al. 2000; Niedzielin et al. 2001; Schultz et al. 2002)
Lactobacillus reuterii	Colitis, Atopic dermatitis, Rotavirus diarrhea, *H. pylori* therapy, Infantile colic, Asthma, Gingivitis, HIV/AIDS	(Madsen et al. 1999; Mukai et al. 2002; Rosenfeldt et al. 2002, 2004; Forsythe et al. 2007; Anukam et al. 2008; Karimi et al. 2009)
Lactobacillus rhamnosus	Atopic dermatitis, Bacterial vaginosis, Diarrhea, *H. pylori* therapy, HIV/AIDS	(Reid et al. 2001; Urbancsek et al. 2001; Rosenfeldt et al. 2002, 2004; Anukam et al. 2008; Irvine et al. 2010)

Lactobacillus rhamnosus GG	Atopic disease, Antibiotic-associated diarrhea, Rotavirus gastroenteritis, *H. pylori* therapy, Dental caries, Ulcerative colitis pouchitis, Irritable bowel syndrome, Crohn's disease, Cystic fibrosis	(Vanderhoof et al. 1999; Armuzzi et al. 2001; Kalliomäki et al. 2001, 2003; Nase et al. 2001; Szajewska et al. 2001; Cremonini et al. 2002; Bruzzese et al. 2004; Montalto et al. 2004; Zocco et al. 2006; Gawrońska et al. 2007)
Lactobacillus salivarius	Colitis, Arthritis, *H. pylori* therapy	(Kabir et al. 1997; McCarthy et al. 2003; Sheil et al. 2004; Peran et al. 2005)
Saccharomyces boulardii	Crohn's disease, *H. pylori* therapy, Diarrhea, Colitis, Amoebiosis	(Guslandi et al. 2000, 2003; Cremonini et al. 2002; Gaón et al. 2003; Mansour-Ghanaei et al. 2003; Duman et al. 2005; Gotteland et al. 2005; Kotowska et al. 2005; Kurugöl and Koturoğlu 2005; Villarruel et al. 2007; Hurduc et al. 2009)

Some of the established microencapsulation techniques have been able to improve the survival of probiotic cells in simulated gastric fluid and simulated intestinal fluid, but improvements in their stability during ambient storage have rarely been reported either in academic research or in commercial development. It is the lack of appropriate stabilizing technology that restricts the use of probiotics in shelf-stable foods.

2.2 Stabilization of probiotics

This review briefly discusses various approaches that are taken to stabilize bacteria. The most commonly used strategy is dehydration and encapsulation in a protective matrix. Optimization of the packaging and the storage conditions are also considered to be vital. Other studies focus on manipulating the cell physiology. These approaches are used independently or in combination to improve the viability of the bacterial cells.

2.2.1 Stabilization using desiccation/dehydration technologies

Drying is a valuable technology for the long term preservation of food materials because decreasing the moisture content slows down the action of enzymes. Various drying technologies have therefore been used, with varying success, to improve the stability of bacteria. In particular, freeze drying, spray drying, fluidized bed drying, vacuum drying, and mixed/two-step drying processes for the stabilization of probiotic bacteria have been studied in recent years and are discussed in some detail below.

2.2.1.1 Freeze drying

Freeze drying is used extensively to preserve biological materials, including bacteria, yeasts, and sporulating fungi, for food and pharmaceutical applications. It is based on the principle of sublimation; the bacteria are frozen, then a vacuum is applied, and the desiccation is carried out by sublimation (Santivarangkna et al. 2007). The advantages

of the freeze drying of bacteria include long viability during storage and ease of transportation. Some limitations are the high cost of the operation and difficulties in scaling up and continuous processing (Santivarangkna et al. 2007).

The step in the freeze drying process that most influences viability is the freezing of the bacteria. The rate of cooling is a critical factor. At an optimum rate of cooling, the cells do not lose water and reach the eutectic point in an amorphous state (Berny and Hennebert, 1991). If the cooling rate is too slow, water will be lost from the cells by osmosis, dehydrating them and preventing freezing. If the cells do not lose water quickly enough to maintain equilibrium, intracellular formation of ice crystals will occur (Mazur, 1977). Significant loss of bacterial cell viability after freeze drying has been attributed to the formation of ice crystals on the cell surface, resulting in an increase in extracellular osmolality, thus leading to dehydration of the cells (Fowler and Toner, 2005), loss of membrane integrity, and denaturation of macromolecules (Franks, 1995; Thammavongs et al. 1996; De Angelis and Gobbetti, 2004). Fonseca et al. (2000) have reported that the size and the shape of the cells are important criteria in their survivability; enterococci (cocci) are more tolerant than lactobacilli (rods) because the membrane damage that is caused by extracellular ice crystal formation during freeze drying is reduced. Loss of viability has also been attributed to peroxidation of the membrane lipids (Brennan et al. 1986; Linders et al. 1997a) and destabilization of the DNA and RNA secondary structures. The recovery rate for freeze dried cultures can be as low as 0.3% (Abadias et al. 2001).

The technology to protect bacterial cells during freeze drying is known as cryopreservation and the compounds used to achieve this protection are called cryoprotectants or cryoprotecting agents (CPAs). The addition of CPAs prior to fermentation or drying

helps the probiotic cells to adapt to the changed environment. The CPA accumulates slowly within the bacterial cells, which helps to reduce the osmotic pressure difference between the inside and the outside of the cells (Kets et al. 1996; Meng et al. 2008). The CPA can be added either to the growth medium or to the drying medium, and its action varies with different bacterial strains. However, certain general compounds, such as nonfat milk solids, lactose, trehalose, glycerol, betaine, adonitol, sucrose, glucose, and dextran, are regarded as being suitable CPAs for many species (Hubálek, 2003; Morgan et al. 2006).

The protection mechanism can be better understood by classifying CPAs into two broad groups: (i) amorphous-glass-forming salts and (ii) eutectic-crystallizing salts. The first group, comprising carbohydrates, proteins, and polymers, acts by imparting very high viscosity at the glass transition, thereby restricting the molecular mobility of the cells. Most of the successful CPAs for probiotic bacteria fall into this group. The second group contains certain eutectic salts that tend to crystallize as the temperature approaches the freezing point; however, instead of providing protection, they have sometimes been reported as being detrimental to the cell membranes (Orndorff and MacKenzie, 1973; Morgan et al. 2006).

CPAs are sometimes classified in a slightly different manner into three groups: (i) highly permeable compounds such as monovalent alcohols, amides, and sulfoxides, (ii) slowly permeable compounds such as glycerol, and (iii) nonpermeable compounds such as mono-, oligo-, and polysaccharides, sugar alcohols, proteins, and polyalcohols. Permeable CPAs bind the intracellular water and prevent dehydration. Non-permeable CPAs form a layer on the cell surface, thus allowing partial outflow of water from the

cell body, reducing the toxic effect of salts, stopping excessive growth of ice crystals, and maintaining their structures (Hubálek, 2003; Saarela et al. 2005).

Trehalose and sucrose have been found to be excellent CPAs against dehydration stress by acting as stabilizers of membranes and proteins and replacers of water from the macromolecular structures (Rudolph and Crowe, 1985; Morgan et al. 2006). Conrad et al. (2000) demonstrated the high cryoprotective efficiency of trehalose; a high survival of *Lactobacillus acidophilus* cells during freeze drying was achieved, probably because of the high glass transition temperature (*T*g) of trehalose. Compounds with a high *T*g can remain relatively immobile at higher temperatures and can help to produce a more stable freeze dried matrix (Crowe et al. 1996; Sun and Davidson 1998; Morgan et al. 2006). Trehalose is also regarded as a good CPA because it can form dihydrate crystals, which leaves the remaining matrix as a glass and does not reduce the *T*g or the stability of the matrix (Crowe et al. 1998). Trehalose and sucrose were shown to be very effective CPAs when *Escherichia coli* DH5 alpha and *Bacillus thuringiensis* HD-1 were subjected to freeze drying (Leslie et al. 1995). In another recent study, trehalose, sucrose, and sorbitol were used as CPAs for lactobacilli and sucrose was found to be the best agent among the group (Siaterlis et al. 2009). However, in one experiment, trehalose achieved a poorer result than the control sample when used in media prior to air drying (Linders et al. 1997b).

Lactose is another popular CPA, has been shown to be more effective than glycerol (Chavarri et al. 1988), and has been found to be effective for certain species, such as *Lactobacillus lactis*, *Escherichia coli*, *Lactobacillus delbrueckii*, and *Saccharomyces cerevisiae*, moderately effective for *Streptomyces tenebrarius*, and completely ineffective for *Spirulina platensis* (Hubálek, 2003). Lactose, and a few other sugar substrates,

has been added to the growth medium for *Lactobacillus delbrueckii*, resulting in a marked improvement in post-drying survival, storage stability, and thermotolerance (Carvalho et al. 2004).

2.2.1.2 Spray drying

In the spray drying process, the atomized liquid is subjected at high pressure to hot air at temperatures up to 200ºC and with an outlet temperature of above 80ºC. This results in the evaporation of moisture after a very short exposure. The production of probiotics by spray drying is advantageous because it is cost effective from an operational point of view, is easy to scale up, is less time consuming than freeze drying, and is a continuous operation with a high production rate (Knorr, 1998). However, as probiotic bacteria are heat-sensitive microorganisms, they are inactivated if subjected to the high temperatures used in the conventional drying process, which has adverse effects on their viability and, in turn, a negative impact on their viability and stability during storage (Gibbs et al. 1999; Kailasapathy, 2002; Madene et al. 2006).

In efforts to reduce cell death during spray drying, Champagne and Fustier (2007) optimized the inlet and outlet temperatures using a combination of spray drying and fluidized bed drying to minimize the heat shock and pre-adaptation of the bacteria to the heat stress prior to drying. Recent studies to obtain higher viability have focused on controlling the outlet air temperature (Table 2-2). However, lower outlet air temperatures result in powder with a high moisture content, which leads to cell death on storage (O'Riordan et al. 2001). Another major limitation of spray drying is the limited choice of shell materials. The shell material has to be water soluble for spray drying to take place, causing immediate release of the core material in the aqueous medium so that controlled release cannot be achieved.

Success of spray drying for encapsulation of probiotics depends on the bacterial strain used as well as the encapsulating material used. O'Riordan et al. (2001) used spray drying technology to coat *Bifidobacterium* cells with starch. There was no significant advantage of microencapsulation during acid exposure and storage, but milder spray drying parameters resulted in less than a 1.0 log reduction in cell viability. Fávaro-Trindade and Grosso (2002) found that *Bifidobacterium lactis* cells were highly resistant to spray drying conditions, with negligible reduction in viability at an inlet temperature of 130ºC and an outlet temperature of 75ºC. In contrast, in the same study, *Lactobacillus acidophilus* cells showed a 2.0 log reduction under similar drying conditions. The encapsulating medium used in this study was cellulose acetate phthalate and very good particle morphology with no pores on the surface was achieved. In another study to protect *Lactobacillus paracasei* during spray drying, Desmond et al. (2002) investigated the encapsulating effect of gum acacia. Cells were grown in both 10% gum acacia and 10% reconstituted skim milk as a control. The cells encapsulated in gum acacia survived 10 times better than the controls, even at a high outlet temperature of 95ºC, the viability during storage at 4ºC was improved 20 fold, and the survival during gastric juice incubation was 100 fold higher. The loss of viability also depends on the type of carrier used. For example, the log reduction in viability was found to be higher in soluble starch than in other carriers, such as gelatin, gum arabic, and skim milk (Lian et al. 2002; Santivarangkna et al. 2007). Picot and Lacroix (2003, 2004) used a modified spray drying process involving a coating of milk fat and denatured whey protein isolate in an attempt to reduce the loss of viability during spray drying. The micronized freeze dried cultures were coated during spray drying and it was concluded that this would be a suitable method to scale up industrially and it is also economical.

Table 2-2 Review of scientific work comparing the viabilities of probiotic bacteria when using spray drying

Bacteria	Inlet air tempera-ture (ºC)	Outlet air tem-perature (ºC)	Matrix	Storage conditions	Study findings	Reference
Bifidobacterium PL1	60		Starch	Screw-capped glass bottles at 19−24ºC for 20 days	Starch encapsulation did not provide protection during storage. Inlet and outlet temperatures of 100/45ºC resulted in less than 1 log reduction after drying	(O'Riordan et al. (2001)
	80					
	100	45				
	≥ 120	60				

Bifidobacterium spp. (30 strains)	175	85−90	Skim milk	Polythene bags and aluminum-coated paper bags at 4, 15 and 25ºC for 90 days	High initial survival following spray drying; storage at 4ºC maintained viability; increased viability loss at 25ºC. The heat and oxygen tolerance varied among closely related species	(Simpson et al. 2005)
Lactobacillus bulgaricus	200	70	Skim milk	Hermetically sealed glass bottles, controlled water activity of 0.11 at 20ºC	The ratio of unsaturated fatty acids to saturated fatty acids decreased during storage as a consequence of lipid oxidation	(Teixeira et al. 1996)
Bifidobacterium longum	100	50−60	Gelatin, starch, skim milk powder,	NA	Increase in outlet air temperature resulted in increased death irrespective of the carrier used	(Lian et al. 2002)

			and gum arabic			
Bifidobacterium infantis Bb-02	160	65	Emulsion containing caseinate, fructo-oligo-saccha-rides, dried glucose syrup, re-sistant starch	Open containers, 25°C and 50% rela-tive humidity for 5 weeks	Microencapsulation protected the viability of bacteria upon storage (> 106 fold) and during simulated gastro-intestinal transit	(Crittenden et al. 2006)
Bifidobacterium	70	47	Pectin and	Closed glass vials, 7	Lactobacillus acidophilus was more	(Oliveira et al.

lactis BI-01 and *Lactobacillus acidophilus*			casein	and 37ºC, water activity of 0.65	viable than Bifidobacterium lactis at both temperatures	2007)

2.2.1.3 Fluidized bed drying

Fluidized bed drying has been widely used in the pharmaceutical industry. It was originally developed for rapid drying but its present applications include granulation, agglomeration, air suspension coating, rotary pelletization, and powder and solution layering. In fluidized bed drying, air travels upward through the bed of particles with sufficient velocity to provide fluid-like behavior, and the freely suspended particles in the air stream are dried by rapid heat exchange and mass transfer. The major advantages of fluidized bed drying are its relatively low operational cost and the high post-drying viability because of the mild heating conditions. Its major limitations are the irregular particle size, the stickiness, the agglomerated particles, the slow drying rate (Santivarangkna et al. 2007), and the uneven distribution of cells in the nonliquid food matrix (Santivarangkna et al. 2007). Fluidized bed drying has been used commercially in the production of baker's yeast and wine yeast (Caron, 1995).

A limited number of studies on the fluidized bed drying of probiotic bacteria and lactic acid starter cultures have been carried out (Santivarangkna et al. 2007). In most, the bacteria were entrapped or encapsulated in a certain matrix before being dried. Linders et al. (1997c) dried *Lactobacillus plantarum* cells that were granulated to pellets using potato starch as the support material. Selmer-Olsen et al. (1999) embedded *Lactobacillus helveticus* cells in calcium alginate gel beads, which were then fluidized bed dried. Mille et al. (2004) fluidized bed dried *Lactobacillus plantarum* and *Lactobacillus bulgaricus*, with the bacteria being uniformly mixed with casein powder before drying. It was found that the bacterial viability was influenced by the water activity of the casein powder; a lower water activity resulted in an increased loss of viability during drying. Strasser et al. (2009) sprayed a concentrated liquid bacterial cell suspension on to fluid-

ized powdered cellulose carrier material. They observed that the addition of protective carbohydrates, such as sucrose and trehalose, prevented a decline in the cell viability of *Lactobacillus plantarum* and *Enterococcus faecium* during fluidized bed drying.

2.2.1.4 Vaccum drying

Vacuum drying is used to preserve heat-sensitive functional ingredients such as vitamins, enzymes, and bacteria, because the process is carried out at low temperature and under reduced pressure. The major advantages of vacuum drying are the high drying rate, the low drying temperature, and the absence of oxygen. The main disadvantage is the longer drying time compared with fluidized bed drying (Santivarangkna et al. 2007).

There have been only a few studies on the vacuum drying of probiotic bacteria. Vacuum drying has usually been applied together with other drying processes to increase its effectiveness. King and Su (1994) dried *Lactobacillus acidophilus* using freeze drying, vacuum drying and the low temperature vacuum drying. The survival ratio from the vacuum-drying (plate temperature, 40-45°C) was too low for the process to be applied in culture preservation. Controlled low temperature vacuum drying (plate temperature, -2°C to 2°C) resulted in survival ratio close to that from freeze drying. The powder produced from low temperature vacuum drying had case hardening, shrinkage and poor rehydration properties.

2.2.1.5 Mixed or two step drying process

In mixed or two-step drying processes, two or more drying systems are combined to minimize cell injury and to improve the bacterial viability. Simpson et al. (2005) found that the combination of spray drying and fluidized bed drying improved the viability of *Bifidobacterium* spp when powders were stored at 25°C. Wolff et al. (1990) found that a combination of vacuum drying and freeze drying was best suited to the dehydration of

concentrated suspensions of *Streptococcus thermophilus.* The survival rate was higher in the vacuum freeze drying process (3.5×10^{11} CFU/g) than in the atmospheric freeze drying process (1.7×10^{11} CFU/g).

2.2.2 Stabilization by encapsulation process

Microencapsulation generally refers to the process of stabilizing an active substance (core) by enclosing it within a physical barrier (shell). The shell consists of either a continuous coating film or a solid wall material, which can release the core at a controlled rate under the direction of specific environmental shifts (Dziezak, 1988; Anal and Singh, 2007). Encapsulation technologies have many useful applications in the food industry, including stabilizing the core material, restraining oxidation, providing prolonged or regulated release, concealing flavors, colors, or odors, improving shelf life, and preventing nutritional loss (Anal and Singh, 2007). The technologies have been extended to encapsulate probiotic bacteria successfully, resulting in enhanced viability of the bacteria by providing protection against environmental stresses (oxygen, acidity in the stomach), facilitating the handling of cells, and allowing controlled dosages (Rokka and Rantamäki 2010). Various types of biopolymer materials, such as starch, dextrin, and whey proteins, have been used as the encapsulation matrix for probiotic bacteria. Table 2-3 summarizes the published research during the last decade on the encapsulation of probiotic bacteria.

Table 2-3 Summary of published research over the last decade on the encapsulation of probiotic bacteria

Encapsulating material	Types of bacteria encapsulated	Product application studied	Research findings	Reference
Alginate	*Lactobacillus acidophilus* (ATCC 43121)	NA	Improved survival in artificial gastric juice, artificial intestinal juice, and heat treatment	(Kim et al. 2008)
Alginate	*Lactobacillus casei*	Ice cream	Improved survival in ice cream during storage over a 3-month period	(Hsiao et al. 2004)
Alginate	*Escherichia coli* GFP+	NA	Showed improved survival of the encapsulated bacteria; also useful in delivering viable bacteria to the intestine	(Song et al. 2003)

Alginate	*Lactobacillus acidophilus* CSCC 2400	NA	Effective protection of the bacteria in simulated gastric conditions and bile salt. Increasing the alginate coating increased the bacterial survivability	(Chandramouli et al. 2004)
Alginate	*Lactobacillus casei* YIT 9018	NA	Improved viability in simulated gastric acid, intestinal juice, simulated bile conditions, and storage at room temperature, 4ºC, and 23ºC for 6 weeks	(Song et al. 2003)
Alginate	*Lactobacillus reuteri* (ATCC 55730)	Fermented sausages	Improved the viability after processing of the dry fermented sausages	(Muthukumarasamy and Holley, 2006)

Alginate	*Lactobacillus casei* (NCDC-298)	NA	Increasing the alginate concentration improved the viability of bacteria in simulated gastric pH and intestinal bile salt solution and the survival of encapsulated cells after heat treatment	(Mandal et al. 2006)
Alginate	*Lactobacillus acidophilus* LA-5 and *Bifidobacterium bifidum* BB12	White-brined cheese	Microencapsulation increased the viability counts of probiotic bacteria during 90 days of storage in brine solution. However, no significant increase in the proteolytic activity was observed because of the restricted release of proteases and peptidases from the capsules during ripening	(Özer and Avni Kirmaci, 2009)

Alginate	*Lactobacillus acidophilus* LA-5 and *Bifidobacterium lactis* BB12	Iranian yogurt milk (doogh)	Improved the viability of probiotic bacteria in refrigerated storage for 42 days	(Mortazavian et al. 2008)
Alginate	*Lactobacillus acidophilus* 2409 and *Bifidobacterium infantis* 1912 and *Lactobacillus casei* 2603	Yogurt	Improved the viability in simulated high gastric conditions, in high bile salt conditions, and during storage for 8 weeks	(Sultana et al. 2000)
Alginate	*Lactobacillus acidophilus* PTCC 1643, *Lactobacillus rhamnosus* PTCC 1637	NA	Double coating in alginate improved the viability in simulated gastric juice and simulated intestinal juice	(Mokarram et al. 2009)
Alginate	Lactic acid bacteria	NA	Improved the viability in the simulated gastric conditions	(Ross et al. 2008)

Alginate	*Lactobacillus acidophilus* LA-1	NA	Improved the survival of cells after heat treatment, homogenization, high sodium chloride concentration, low pH, and high bile salt concentration	(Sabikhi et al. 2010)
Alginate	*Bifidobacterium animalis* subsp. *lactis* BB12	NA	Improved the survivability during exposure to nisin and simulated gastric juice; refrigerated storage at 4ºC for 28 days	(González-Sánchez et al. 2010)
Alginate	*Lactobacillus acidophilus* LA-5 and *Bifidobacterium bifidum* BB12	Kasar cheese	Improved the viability during storage for 90 days	(Özer et al. 2008)
Alginate	*Lactobacillus casei* (Lc-01) and *Bifidobacterium lactis*	Ice cream	Improved the survivability during storage at −20ºC for 180 days	(Homayouni et al. 2008)

	(BB12)			
Alginate	*Lactobacillus acidophilus* (ATCC-314)	Yogurt	Encapsulated *Lactobacillus acidophilus* suppressed the incidence of colon tumor	(Urbanska et al. 2007)
Compressed Alginate and Hydroxypropyl cellulose	*Lactobacillus acidophilus* (ATCC 4356)	NA	Improved the viability on storage at 25ºC for 30 days. The cell viability decreased with the compression pressure	(Chan and Zhang, 2002)
Alginate and Poly-L-lysine and Palm oil	*Lactobacillus rhamnosus*, *Bifidobacterium longum*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus paracasei*,	NA	Protected the probiotic bacteria from acid and bile salts. Poly-L-lysine provided better protection than alginate under acidic conditions	(Ding and Shah, 2009a, 2009b)

	Bifidobacterium lactis Bl-04, and *Bifidobacterium lactis* BI-07			
Alginate−chitosan	*Lactobacillus acidophilus* (ATCC-314)	Yogurt	Served as a suitable carrier and protected the bacteria in simulated gastric fluid and simulated intestinal fluid	(Urbanska et al. 2007)
Alginate-coated matrix and whey protein	*Lactobacillus plantarum* (Lp299v, LpA159, Lp800)	NA	Whey protein coating of alginate beads significantly improved the viability of the bacteria in simulated gastric and intestinal fluids	(Gbassi et al. 2009)

Alginate−gelatin	*Lactobacillus casei* ATCC 393	NA	Relative humidity had a minor effect on the characteristics of the microcapsules. Improved viability of the encapsulated cells in simulated gastric fluid and simulated intestinal fluid	(Li et al. 2009)
Alginate–poly-L-lysine−alginate	*Lactobacillus plantarum* 80	NA	Showed the effectiveness of the coating in a simulated human gastrointestinal medium	(Lian et al. 2003)
Alginate, N-Tack, N-Lok, and Hylon VII	*Lactobacillus acidophilus*	NA	Improved the viability on refrigerated storage for 4 weeks. Fluidization was found to be the most effective process	(Goderska and Czarnecki, 2008)

Alginate, Poly-L-lysine, Chitosan	*Lactobacillus acidophilus* 547, *Lactobacillus casei* 01 and *Bifidobacterium bifidum*	NA	Provided the best protection for *L. acidophilus* and *L. casei* in bile salt solution, simulated gastric juice, and intestinal juice with or without chitosan. *B. bifidum* did not survive in the acidic conditions prevalent in the stomach	(Muthukumarasamy and Holley, 2006)
Alginate, Poly-L-Lysine, Chitosan, and Prebiotics	*Lactobacillus acidophilus* CSCC 2400 or CSCC 2409	Yogurt	Improved the survival of the bacteria in vitro under acidic and bile salt conditions and in storage of the yogurt for 6 weeks	(Iyer and Kailasapathy, 2005)
Alginate, κ-Carrageenan	*Bifidobacterium bifidum* and *Lactobacillus acidophilus* LA-5	White-brined cheese	Encapsulation improved the viability of the probiotic cells in the cheese during ripening	(Özer et al. 2009)

Alginate/Alginate + starch/κ-Carrageenan + locust bean gum/Xanthan gum + gellan gum	*Lactobacillus reuteri* 1063 (ATCC 53608)	NA	Greater protection in simulated gastric juice and simulated bile juice by both extrusion and phase separation	(Muthuku-marasamy and Hol-ley, 2007)
Algi-nate−methylcellulose	*Bacillus polyfermenticus* SCD	NA	Improved survival in artificial gas-tric juices and artificial bile salt	(Kim et al. 2006)
Alginate−chitosan	*Bifidobacterium animalis* subsp. lactis	NA	Improved the controlled release of bifidobacteria in simulated gastric juice	(Liserre et al. 2007)
Alginate−pectin	*Lactobacillus casei* LB C81	Yogurt	Encapsulation of beads with algi-nate and pectin blends in 1:4 and 1:6 ratios improved the viability in simulated gastric juice, in bile salt solution, and during storage for 20	(Sandoval-Castilla et al. 2010)

			days at 4ºC	
Bacterial cellulose, Nata, Calcium alginate, Skim milk	*Lactobacillus bulgaricus* NCIM2056, *Lactobacillus plantarum* NCIM 2083, *Lactobacillus delbrueckii* NCIM 2025, *Lactobacillus acidophilus* NCIM 2902, and *Lactobacillus casei* NCIM 2651	NA	Bacterial cellulose as a CPA and immobilization support for probi-otic lactic acid bacteria. Storage study at 4 and 30ºC for 60 days	(Jagannath et al. 2010)
Chitosan−alginate	*Lactobacillus bulgaricus*	NA	Improved the survival in simulated gastric juice and during refrigerated storage at 4ºC	(Shima et al. 2006)

Gelatin, Gum arabic, Soluble starch, and Skim milk	*Bifidobacterium longum* B6 and *Bifidobacterium infantis* CCRC 14633	NA	Increased the viability of the microencapsulated cells in simulated gastric juice and bile solution	(Lian et al. 2003)
Gellan−alginate	*Bifidobacterium bifidum*	NA	Improved the protection during pasteurization and in simulated gastric juice	(Chen et al. 2007)
Genipin−gelatin	*Bifidobacterium lactis* BB12	NA	Increasing the genipin concentration improved the stability in simulated gastric juice. The presence of the gastric enzyme pepsin reduced the bead stability	(Annan et al. 2007)
Hexaglyceryl condensed ricinoleate	*Lactobacillus acidophilus* (JCM1132)	NA	The inner phase volume ratio and the median diameter of the oil droplets affected the viability in simu-	(Shima et al. 2006)

			lated gastric juice	
Hydrolyzed potato starch and Cocoa butter	*Bifidobacterium longum* 2C, *Bifidobacterium longum* 46, *Bifidobacterium adolescentis* VTT, and *Bifidobacterium lactis* BB12	Fermented and nonfermented oat drink	Encapsulation in cocoa butter improved the culturability whereas hydrolyzed potato starch had no effect when stored for 4 weeks	(Lahtinen et al. 2007)
Maize starch	*Bifidobacterium* PL1	Commercial muesli preparation and dry malted beverage powder	Acid tolerance assay and storage in food for 14 days. Advocated that modified starch is not suitable for the encapsulation of probiotic bacteria	(O'Riordan et al. 2001)

Pectin starch, Gelatin, and Starch	*Lactobacillus reuteri* (DSM 20016)	Apple juice	Encapsulated material. Determined the process stability and the storage stability at 5ºC for 120 days in simulated gastric juice	(Weissbrodt and Kunz, 2007)
Rennet-gelled milk protein	*Lactobacillus paracasei* subsp. paracasei F19 and *Bifidobacterium lactis* BB12	NA	Improved the bacterial survival in simulated gastric conditions because of the buffering action of the protein	(Heidebach et al. 2009a)
Sesame oil	*Lactobacillus delbrueckii* subsp. bulgaricus	NA	Improved the viability of the bacteria in simulated high acid gastric conditions and simulated bile salt conditions	(Hou et al. 2003)
Sodium alginate and Hydroxypropylcellu-	*Lactobacillus acidophilus* (ATCC 4356)	NA	Improved the survival in simulated gastric fluid	(Chan and Zhang, 2005)

lose				
Supercritical carbon dioxide interpolymer complex	*Bifidobacterium longum* Bb-46	NA	Improved the survival of bacteria in simulated gastric and intestinal fluids	(Thantsha et al. 2009)
Transglutaminase-induced casein gel	*Lactobacillus paracasei* subsp. *paracasei* F19 and *Bifidobacterium lactis* BB12	NA	Provided protection in simulated gastric juice without pepsin	(Heidebach et al. 2009b)
Transglutaminase-induced casein gel	*Lactobacillus paracasei* subsp. *paracasei* F19 and *Bifidobacterium lactis* BB12	NA	Improved the viability during storage at 4 and 25ºC for 90 days at 11 and 33% relative humidity	(Heidebach et al. 2010)
Whey protein isolate	*Lactobacillus rhamnosus* R011	Semisweet biscuits, refrigerated vegetable juice, and frozen fruit juice	Protected the cells during a short heat treatment. Storage of the product for 3 weeks	(Reid et al. 2007)

Whey protein−pectin and alginate	*Bifidobacterium bifidum* R071	NA	Improved the viability in simulated gastric conditions and simulated high bile salt conditions	(Guérin et al. 2003)

2.2.2.1 Encapsulating materials

Microencapsulation in tailored carriers composed of nontoxic biopolymers provides mechanical protection and allows probiotic microorganisms to be used in several food products. The encapsulating material serves two major functions: (i) to provide a definite physical structure for delivery in the food systems and (ii) to protect the living cells during harsh processing, during storage, and in the low pH environment in the stomach (Ding and Shah, 2009a, 2009b). The materials that are most commonly used for probiotic encapsulation are (i) polysaccharides − carrageenan and alginate (extracted from marine algae), starch and its derivatives, and bacterial exopolysaccharides such as gellan gum and xanthan gum, (ii) proteins such as casein, whey protein (milk proteins), and gelatin, and (iii) fats and oils such as cocoa butter, sesame oil, and hexaglyceryl condensed ricinoleate. Brief discussions on each type are presented below.

2.2.2.1.1 Polysaccharides

2.2.2.1.1.1 Carrageenan

κ-Carrageenan is a natural polysaccharide that is made up of repeating sulphated galactose units and 3,6-anhydrogalactose, which are joined together by alternating α-(1,3) and β-(1,4) glycosidic linkages. It is extracted from red marine algae and is commonly used as an emulsifier in food products. The gelation of carrageenan is a temperature-dependent process that is induced by temperature changes. Embedded beads are formed by dispersing a suspension of cells and a heat-sterilized polymer mix into a warm organic phase (40−45ºC) and gelation occurs by cooling to room temperature (Audet et al. 1988). The beads are seasoned by soaking in potassium chloride solution. The potassium ions stabilize the gel and prevent swelling; however, potassium chloride affects the growth of lactic acid bacteria (Audet et al. 1988). Moreover, the addition of locust bean

gum to κ-carrageenan, especially at a ratio of 2:1, improves the rigidity (Miles et al. 1984; Audet et al. 1990). There is a synergistic interaction between κ-carrageenan and locust bean gum, which improves the rheological properties of the resulting gel (Miles et al. 1984; Arnaud et al. 1988 a, 1988 b). Doleyres et al. (2002a, 2004) demonstrated continuous production of a mixed culture (*Bifidobacterium longum* and *Lactococcus diacetylactis*) that was embedded in κ-carrageenan and locust bean gum gel beads.

2.2.2.1.1.2 Alginate

Alginate is a linear heteropolysaccharide of 1−4-linked β-D-mannuronic acid and α-L-guluronic acid residues, which varies in composition based on the source of extraction (Smidsrod and Skjak-Braek, 1990). Microencapsulation in calcium alginate beads for drug delivery systems, enzyme immobilization, and immunoprotective containers in cell transplantation has been investigated. Entrapment with calcium alginate beads has frequently been used for the immobilization of lactic acid bacteria because of the simple method of immobilization and the advantage of being nontoxic to cells. Gel formation occurs when in contact with calcium and multivalent cations (Prevost and Divies, 1992; Iyer and Kailasapathy, 2005).

Compared with free cells, encapsulating bacteria in alginate beads increased survival during heat treatment, during homogenization, at high sodium chloride concentration, at low pH in simulated gastric juice, at high bile salt concentration in simulated intestinal fluid, and during storage (Song et al. 2003; Mandal et al. 2006; Ross et al. 2008; Mokarram et al. 2009; González-Sánchez et al. 2010; Sabikhi et al. 2010). Furthermore, several researchers have shown improved viability of alginate-encapsulated cells in ice cream (Homayouni et al. 2008), cheese (Özer et al. 2008, 2009; Özer and Avni Kirmaci 2009),

yogurt (Sultana et al. 2000; Urbanska et al. 2007), Iranian yogurt milk (doogh) (Mortazavian et al. 2008), and fermented sausages (Muthukumarasamy and Holley, 2006).

Gåserød et al. (1999) observed that the presence of calcium-chelating agents such as phosphate, citrate, and lactate or non-gelling cations such as sodium and magnesium destabilizes calcium alginate gels. Alginate capsules (or microcapsules) are stable at low pH, but swell in weak basic solutions followed by disintegration and erosion (Lee et al. 2003). Alginate polycation membranes have been used for encapsulation in an attempt to overcome the limitations of uncoated alginate membranes. They provide the added advantage of increased membrane strength and reduced permeability (Gåserød et al. 1999). Liserre et al. (2007) studied alginate−chitosan beads for their controlled release of *Bifidobacterium animalis* in simulated gastric juice. Muthukumarasamy and Holley (2006) found that encapsulation of *Lactobacillus acidophilus* and *Lactobacillus casei* in an alginate−chitosan membrane provided protection against artificial gastric and intestinal juices. Iyer and Kailasapathy (2005) and Urbanska et al. (2007) studied the microencapsulation of *Lactobacillus acidophilus* in an alginate−chitosan membrane and its application in yogurt. They reported that the carrier matrix could protect bacteria in simulated gastric fluid, in simulated intestinal fluid, and under refrigerated storage at 4°C. Other combinations with alginate that have been investigated are alginate−pectin (Sandoval-Castilla et al. 2010), alginate−whey protein (Gbassi et al. 2009), alginate−gelatin (Li et al. 2009), alginate−hydroxypropyl cellulose (Chan and Zhang, 2002), and alginate−gellan gum (Chen et al. 2007).

2.2.2.1.1.3 Starch and its derivatives

Starch is the storage form of carbohydrate in plants. It is composed of amylose, a linear polysaccharide, and amylopectin, a branched chain polymer. Amylose consists of D-

glucopyranose residues and is joined by α-(1,4) linkages. In native starches these linkages are resistant to pancreatic α-amylases but are easily degraded by the colonic microflora, which makes them useful for the delivery of bioactive compounds in the colon. Starch also offers the additional advantage of being cheap and readily available.

Native starch can easily be modified to remove some of its unsuitable characteristics that limit its application. Starch is modified by introducing a chemical substituent (hydrophilic or hydrophobic) via a reaction with the hydroxyl groups in the starch molecule. Resistant starch is a type of modified starch that is not digested in the small intestine and reaches the colon, where it is used by the colonic microflora to produce short chain fatty acids (Goñi et al. 1996; Anal and Singh, 2007). High amylose corn starch containing 20% resistant starch has been found to be suitable for enteric delivery (Dimantov et al. 2004) and the addition of resistant starch to an encapsulation matrix has been found to improve the viability of *Lactobacillus casei* (Sultana et al. 2000) and *Bifidobacterium lactis* (Homayouni et al. 2008).

2.2.2.1.1.4 Gellan gum and xanthan gum

Gellan gum is an anionic bacterial exopolysaccharide that is derived from *Sphingomonas elodea*. It is a linear tetrasaccharide, with a 500 kDa backbone of [→3) Glc (β1→4) GlcA (β1→4) Glc-(β1→4) L-Rha (β1→], where Glc is glucose, GlcA is glucuronic acid, and Rha is rhamnose, with side groups consisting of O-acetyl and L-glycerate substituents (Pollock, 1993). Its functionality depends mostly on the degree of acylation and its constituent ions. Its most unique function is its ability to hold small particles in suspension without increasing its viscosity significantly (Baird and Pettitt, 1991). Also, gellan gum is not easily degraded by the action of enzymes (Baird and Pettitt 1991; Lee, 1996) and is resistant to an acidic environment (Sun and Griffiths, 2000).

Gellan gum forms very firm and brittle gels but melts easily in the mouth, releasing water and any flavor from its gel network (Sun and Griffiths, 2000). Although gellan gum itself is able to form good microcapsules when gelled in the presence of calcium ions, this process needs a preheating of up to 80ºC for 1 h (Sanderson, 1990), which is not suitable for heat-sensitive core materials such as probiotics. Therefore, for encapsulation purposes, gellan gum has always been used in combination with another gum, such as xanthan gum (Sun and Griffiths, 2000; McMaster et al. 2005; Muthukumarasamy et al. 2006), or a few sequestrants, such as sodium citrate, sodium metaphosphate, and EDTA (Camelin et al. 1993).

In several studies, a combination of gellan gum and xanthan gum has been used to encapsulate probiotic bacteria. Norton and Lacroix (1990) reported that the gellan gum−xanthan gum beads were useful for encapsulating *Bifidobacterium* cells and for protecting them from acid injury. Sun and Griffiths (2000) reported improved viability of *Bifidobacterium infantis* cells embedded with gellan gum−xanthan gum when exposed to simulated gastric juice. Furthermore, they reported improved viability of the encapsulated cells, compared with the free cells, when stored in pasteurized yogurt for 5 weeks at 4°C.

2.2.2.1.2 Proteins

2.2.2.1.2.1 Milk Proteins

Milk proteins provide good functional properties that promote their use as shell or matrix materials for microencapsulation. They are relatively inexpensive, are widely available in nature, possess good sensory properties, are highly soluble, have low viscosity in solution, and have good emulsification and film-forming properties (Forrest et al. 2005; Day et al. 2007; Semo et al. 2007; Sandra et al. 2008; Livney, 2010). The protein medi-

um surrounding the bacterial cells provides a very good buffer in comparison with plant hydrocolloid materials. Milk proteins help to increase the pH of gastric juice significantly and, thus, increase the survivability of the encapsulated bacteria (Charteris et al. 1998; Corcoran et al. 2004). Milk proteins have excellent gelation properties, which are appealing to the microencapsulation industry. Their gelation results from the unfolding of the protein and subsequent aggregation. Noncovalent bonds stabilize the gel network and covalent bonds, such as disulfide bridges, may also participate in the stabilization process (Li et al. 2006).

Casein, in the form of an aqueous solution of sodium caseinate, can be coagulated and gelled by the actions of enzymes, such as rennet and transglutaminase (Heidebach et al. 2009a, 2009b), by cross-linking with glutaraldehyde (Latha et al. 2000), or by slow acidification with glucono-δ-lactone (Lucey et al. 1997). The acid gelation of casein is based on the principle that the net charge on the protein is zero at its isoelectric point. Thus, repulsive forces are minimal, resulting in easy movement and the aggregation of the molecule. Rennet-induced gelation is based on the proteolytic cleavage of the hydrophilic hairy outer layer of κ-casein, resulting in micellar aggregation. Transglutaminase-induced gelation takes place by the catalysis of the acyl-transfer reaction, resulting in the formation of molecular cross-links in the protein (Cho et al. 2003). The functional and physicochemical properties of the transglutaminase-cross-linked proteins depend on factors such as enzyme concentration, incubation time, and the type and concentration of the proteins.

Sodium caseinate appears to offer the ideal physical and functional properties for microencapsulation because of its amphiphilic and emulsifying characteristics (Hogan et al. 2001; Madene et al. 2006). Heidebach et al. (2009b) developed transglutaminase-

catalyzed gelation of casein suspensions containing the probiotic cells *Lactobacillus paracasei* and *Bifidobacterium lactis*. The aggregated casein mass was found to protect the microorganisms from damage in simulated gastric conditions. In another study, Heidebach et al. (2009a) used rennet to induce the gelation of skim milk concentrates and determined their potential to encapsulate probiotic bacteria. Nag et al. (2011) used sodium caseinate and gellan gum mixture gelled by gradually decreasing the pH with glucono-δ-lactone (GDL) to encapsulate *Lactobacillus casei* cells. The capsules provided enhanced protection in simulated gastric juice and bile salts.

The formation of whey protein gels at above 75°C is due mainly to the gelation of β-lactoglobulin, which comprises 50% of the total whey protein (Kilara and Vaghela, 2004) and is the most abundant whey protein in bovine milk. The heat treatment makes this process unsuitable for encapsulating many heat-sensitive materials such as probiotic bacteria (Chen et al. 2006). The cold gelation of whey proteins is a potential solution to this problem (Barbut and Foegeding, 1993; Maltais et al. 2005; Heidebach et al. 2009a). As native β-lactoglobulin is resistant to gastric digestion (Miranda and Pelissier, 1983), peptic hydrolysis (Guo et al. 1995), and tryptic hydrolysis (Reddy et al. 1988), it is a suitable encapsulating material for probiotic delivery. It also possesses very good oxygen barrier properties (Kim et al. 1996).

An ionotropic gelation technique has been used to encapsulate probiotics with whey protein isolate (Kailasapathy and Sureeta, 2004; Ainsley Reid et al. 2005). The addition of cations, in the form of calcium, shields electrostatic charges on the surface of the proteins and allows the molecules to move close together. The resulting aggregation and gelation are due to the absence of electrostatic repulsion. As this method is carried out at room temperature, it is suitable for encapsulating heat-sensitive probiotic microorgan-

isms. Guérin et al. (2003) used a combination of alginate, pectin, and whey protein to encapsulate *Bifidobacterium* cells. The capsules offered much better protection than free cells against the low pH of simulated gastric juice and the bile salts present in simulated intestinal fluid. Gbassi et al. (2009) noted that whey proteins are a convenient, cheap, and efficient material for coating calcium alginate beads loaded with *Lactobacillus plantarum*. The whey protein coating significantly improved bacterial survival in a simulated gastric environment. Picot and Lacroix (2004) observed the potential of embedded *Bifidobacterium longum* and *Bifidobacterium breve* in whey-protein-based microcapsules to survive the harsh environmental conditions in the stomach and in acidic products such as yogurt.

2.2.2.1.2.2 Gelatin

Gelatin is produced from denatured collagen and contains significant amounts of hydroxyproline, proline, and glycine. It is useful as a thermally reversible gelling agent for encapsulation. Gelatin has good membrane-forming ability, has good biocompatibility, and is nontoxic. One limitation of the use of gelatin in the hydrogel matrix is its low network rigidity, but this can be improved by adding cross-linking agents. Lian et al. (2003) reported that the encapsulation of *Bifidobacterium longum* B6 and *Bifidobacterium infantis* CCRC 14633 in gelatin resulted in increased viability of the cells in a simulated gastric juice and bile solution. Li et al. (2009) found that microencapsulation using alginate−gelatin beads improved the survival of *Lactobacillus casei* ATCC 393 during gastrointestinal transit. Annan et al. (2007) encapsulated *Bifidobacterium lactis* BB12 in genipin−gelatin capsules and observed that increasing the genipin concentration improved their stability in simulated gastric juice. Genipin is derived from an iridoid glycoside called geniposide present in the fruit of *Gardenia jasminoides* is a natural crosslinker for protein.

2.2.2.1.3 Fats and oils

Fats and oils possess good oxygen and moisture barrier properties. Their presence on a surface increases its hydrophobicity, thus preventing moisture uptake. Lahtinen et al. (2007) observed that encapsulation of *Bifidobacterium longum*, *B. adolescentis*, and *B. lactis* in cocoa butter improved the survivability of the cells when stored for 4 weeks at 4°C in non-fermented oat drink. The average weekly reduction in the plate counts of the encapsulated cells (0.7 log CFU/ml/week) was significantly less than in the free control cells (1.3 log CFU/ml/ week). Similarly, Hou et al. (2003) found that the encapsulation of *Lactobacillus delbrueckii* subsp. *bulgaricus* in sesame oil improved its viability under simulated gastric and intestinal conditions.

2.2.2.2 Encapsulation technologies

Probiotics are most commonly microencapsulated using emulsion and extrusion techniques. In any microencapsulation technique, the size of the capsule beads is important because it influences the sensory properties, governing its use in foods. The extrusion technique results in beads of uniform size (2000−4000 µm), whereas alginate beads made using the emulsion technique vary in size from 20 to 2000 µm but are smaller than extruded alginate beads. Coacervation for probiotic delivery has also been investigated. However, other microencapsulation techniques such as centrifugal extrusion, spray chilling, co-crystallization, and molecular inclusion, which have been utilized to encapsulate food ingredients such as vitamins, flavors, fruit juices, and essential oils, have found limited application with probiotics. The major microencapsulation techniques used for probiotics are discussed in further detail.

2.2.2.2.1 Emulsion techniques

This method involves taking a small volume of cell−polymer suspension (discontinuous phase) and adding it to a large volume of a vegetable oil (continuous phase), such as soybean oil, sunflower oil, canola oil, or corn oil. The mixture is homogenized to form a water-in-oil emulsion. Then, the water-soluble polymer is insolubilized (cross-linked) by slowly adding a solution of calcium chloride, resulting in phase separation. The dispersed phase encapsulates the probiotic bacteria as the core material (Krasaekoopt et al. 2003). Finally, the beads are collected using filtration or mild centrifugation (Sheu and Marshall, 1993). One major advantage of the emulsion technique is that it can easily be scaled up for commercial production. Also, the microspheres are smaller in size and of more uniform shape than those produced by the extrusion technique. A major disadvantage is the higher operational cost associated with the use of vegetable oil in the process. Most studies on the emulsion technique for encapsulation have used sodium alginate as the carrier material. The particle size distribution of the microcapsules is controlled by the speed of agitation, the homogenization parameters, and the type of emulsifier. Song et al. (2003) made an emulsion of sodium alginate in corn salad oil by passing it through a microporous glass membrane; they then used the emulsion to encapsulate *Lactobacillus casei* YIT 9018 cells. The encapsulated cells had a small particle size, and provided increased stability in artificial gastric and bile salt solutions and during storage at different temperatures. Materials other than alginate that are commonly used in the emulsion technique are a mix of κ-carrageenan and locust bean gum (Audet et al. 1988), chitosan (Groboillot et al. 1993), gelatin (Hyndman et al. 1993), and cellulose acetate phthalate (Rao et al. 1989). Adhikari et al. (2000) used 2% κ-carrageenan and 0.9% sodium chloride dispersed into vegetable oil and emulsified the mix with Tween 80, followed by immobilization with potassium chloride. Free and encapsulated

Bifidobacterium longum cells were tested for their resistance in an acidic yogurt medium during refrigerated storage for 30 days. The encapsulated cells had a significantly better survival rate (> 70.5−78%) than the free cells. In another study Nag et al. (2011) successfully encapsulated *Lactobacillus casei* into a gel matrix (comprising of sodium caseinate and gellan gum) by oil in water emulsion. The capsules were found to provide enhanced protection to cells under simulated gastric juice and bile salt conditions.

2.2.2.2.2 Extrusion techniques

Extrusion is the oldest and most common technique for converting hydrocolloids into microcapsules (King, 1995). The gelation of hydrocolloids, such as alginate, carrageenan, and pectin, in the presence of minerals such as calcium and potassium has been used successfully to entrap probiotic bacteria using extrusion. The chemical explanation is that the calcium and potassium ions bind to the multiple free carboxylic radicals, thereby forming gels (Champagne and Fustier, 2007). Different concentrations of alginate have been used to form tiny gel particles. In this process, the hydrocolloid and the probiotic mixture are dripped into the hardening solution (calcium chloride) through a syringe or nozzle (Eikmeier and Rehm, 1987; Champagne et al. 1992; Desai et al. 2005). The viscosity of the sodium alginate solution, the composition of the alginate, the distance between the syringe and the calcium chloride collecting solution, and the orifice diameter of the extruder influence the size and sphericity of the beads (Smidsrod and Skjak-Braek, 1990). Extrusion is the preferred technique for coating volatile flavors and oils. It increases the shelf life of compounds by inhibiting oxygen diffusion through the matrix (Gouin, 2004; Desai and Park, 2005a, 2005b; Madene et al. 2006). In a recent study, Li et al. (2009) microencapsulated *Lactobacillus casei* cells in a mix of sodium alginate and gelatin using an extrusion process. This combination successfully protected the cells during gastrointestinal transit, but the beads were relatively large in

size, with a mean diameter of 1.1 ± 0.2 mm. The encapsulated cells showed higher levels of cell viability as compared to the free cells (non-encapsulated bacteria). Homayouni et al. (2008) microencapsulated probiotics into calcium alginate beads in the presence of resistant starch and incorporated them into ice cream. After storage for 180 days at −20°C, the survival rate of the encapsulated cells was 30% higher than that of the free cells. A major advantage of the extrusion process is its shell−core character. The encapsulated material is completely covered and protected by the wall material, and residual or surface core material is removed by a dehydrating liquid, generally isopropyl alcohol (Gibbs et al. 1999; Desai et al. 2005). The cost involved in the production of encapsulated particles is lower for the extrusion technique than for the emulsion technique.

However, it is difficult to scale up the extrusion technique for commercial application. The bead formed by the extrusion technique is larger in size than that produced by the emulsion technique and is not of uniform size and shape.

2.2.3 Stabilization by manipulating the cell physiology

Greater understanding of the physiology of bacterial cells can help to improve the desirable characteristics of bacteria, such as enhanced acid and bile tolerance. Recently, modification of the growth conditions to enhance the stress tolerance level has been used to improve the robustness of probiotic cultures. Optimization of the growth phase medium and the cell harvesting conditions are also potential ways of improving the stability of the cultures. Intrinsic tolerance of the bacterial strains, bacterial stress adaptation, and growth phase and cell harvesting conditions as tools for improvement of bacterial stability are discussed below.

2.2.3.1 Intrinsic tolerance of culture/strain selection

The inherent vulnerability of bacterial cells varies between different genera as well as between distinct species of the same genus. Compared with lactobacilli, streptococci have been found to be more resistant to heat. Consequently, the size and the shape of the cells have become important factors in influencing bacterial viability (Clark and Martin, 1994). As example of species to species variation, various species of *Bifidobacterium* show considerable variability in the tolerance to heat and oxygen (Simpson et al. 2005). Moreover, *Bifidobacterium longum* has been found to have high bile salt tolerance, as high as 4% (Clark and Martin, 1994), which is notably higher compared to many others *Bifidibacterium* species. However, there is no rational explanation for the variation in viability. The selection of bacterial culture strains based on their ability to resist low pH, bile salt concentrations, heat, desiccation, and oxygen is beneficial for the preservation of viability in food applications.

2.2.3.2 Stress adaptation techniques

Bacteria possess an inherent ability to withstand harsh conditions and sudden environmental changes by inducing stress proteins. In particular, bacteria that can withstand an adverse environment can subsequently survive near-fatal conditions. Stress adaptation is defined as an increase in an organism’s tolerance to deleterious factors on exposure to sub-lethal stress (Hendrick and Hartl, 1993; Gottesman et al. 1997; Van de Guchte et al. 2002). The resistance offered by a bacterial defence system can be grouped into two categories: (i) a specific system that allows survival against a challenged dose of the same agent, such as heat, osmotic stress, oxygen, and low pH (Desmond et al. 2001; Gouesbet et al. 2001); (ii) pre-adaptation to a stress condition, which can allow the cells to resist diverse environmental stresses and is known as a cross-adaptive response (Kim et al. 2001). Pre-adaptation occurs as a result of the convergence of reactive genes to

distinct kinds of injury, e.g., heat, osmotic stress, oxygen, and low pH (Crawford and Davies, 1994). Recent studies have focused on pre-adaptation to heat, osmotic stress, oxygen, and low pH to improve the stress tolerance level of bacteria. Three of the main stresses are explained below.

2.2.3.2.1 Heat stress

Heat stress in bacteria results from an abrupt increase in temperature. Cells subjected to high temperatures often show decreased protein stability, and membranes and nucleic acids are affected. High temperature also disturbs the transmembrane proton gradient, which leads to a decrease in intracellular pH (Hecker et al. 1996; Lindquist and Craig, 1988). Heat tolerance is a process whereby microorganisms exposed to sub-lethal heat treatment acquire the ability to withstand subsequent lethal heat challenges (Girgis et al. 2003). Heat-induced thermotolerance has been observed in several *Lactobacillus* species. For example, the application of heat stress resulted in the expression of heat-stress proteins such as GroES, GroEL, htrA, HcrA, GrpE, DnaK, DnaJ, GroEL, and DnaK in *Lactobacillus helveticus* (Hecker et al. 1996; Broadbent et al. 1998; Smeds et al. 1998), *L. acidophilus* (Zink et al. 2000), *L. bulgaricus* (Gouesbet et al. 2001, 2002), *L. sakei* (Schmidt et al. 1999; Stentz et al. 2000), and *L. johnsonii* (Walker et al. 1999; Zink et al. 2000). The stress proteins help to preserve the native conformation of the cellular proteins and minimize denaturation. They also promote proper assembly of the proteins and prevent unfolding and aggregation (Craig et al. 1993). Moreover, in response to heat stress, there is a change in the lipid composition of the bacterial membrane, e.g., a substantial increase in the levels of C19:0 cyclopropane fatty acids. A lower ratio of saturated fatty acids to unsaturated fatty acids has been observed (Broadbent and Lin, 1999).

2.2.3.2.2 Osmotic stress

In response to osmotic stress, bacteria accumulate nontoxic low molecular weight compounds, called compatible solutes, within their cells. The accumulation of compatible solutes by lactic acid bacteria has been found to be beneficial during drying and osmotic stress (Kets et al. 1996) and to improve the viability on storage.These compatible solutes allow the cell to retain positive turgor pressure by reducing the osmotic pressure difference between the internal environment and the external environment, thereby prolonging the viability of the cells (Kets et al. 1996). They function to balance the osmolality with the extracellular environment, to enhance enzyme stability at low water activity, and to maintain the integrity of the cellular membrane during dissociation (Kets and De Bont, 1994).

Microorganisms have been observed to accumulate compatible solutes, such as amino acids, quaternary amines (e.g., glycine, betaine, and carnitine), saccharides, and polyols, to protect them against decreasing water activity and to help the cell to acclimatize against osmotic stress (Poolman and Glaasker, 1998). Glycine betaine is identified as an intracellular osmolyte that protects *Lactobacillus acidophilus* from osmotic stress (Hutkins et al. 1987). Prasad et al. (2003) found that the robustness of *Lactobacillus rhamnosus* HN001 can be enhanced by osmotic stress adaptation to cope with industrial practices such as desiccation and rehydration and the storage environment. They proposed that the process/mechanism associated with the protective osmotic shock effect acquired by dried *L. rhamnosus* HN001 is stress proteins, along with glycolysis-related machinery and other stationary phase proteins and regulatory factors. Desmond et al. (2001) noted the cross stress tolerance to heat in *Lactobacillus acidophilus* upon exposure to osmotic stress (0.3 mol sodium chloride/L for 30 min). They also observed that the survival rate was 16 times higher after exposure to osmotic stress (0.3 M NaCl for

30 minutes) than for the untreated control culture each in reconstituted skim milk at 37°C. Exopolysaccharide production acts as a survival approach to safeguard bacteria in damaging environments such as against osmotic stress (Ruas-Madiedo et al. 2002).

2.2.3.2.3 Acid stress

Acid tolerance and adaptation increase the survival of probiotic bacteria in the gastrointestinal tract. Lactic acid is the major threat to the cell in the low pH environment in fermented dairy products. The organic acids remain protonated and unchanged and can easily pass into the cell through the cell membrane (Girgis et al. 2003). Bacteria possess several defensive mechanisms, which confer protection against acid injury. These defensive mechanisms perform many functions to maintain viability under low pH conditions. Some of the important functions are: (i) translocation of protons to the environment (Kobayashi et al. 1984, 1986; Nannen and Hutkins, 1991); (ii) production of acid-neutralizing ammonia from arginine (Marquis et al. 1987); (iii) amino acid decarboxylation (Molenaar et al. 1993); (iv) decarboxylation of oxaloacetate (Ramos et al. 1994; Lolkema et al. 1995; MartyTeysset et al. 1996). Shah and Ravula (2000) utilized the acid stress adaptation response of *Lactobacillus acidophilus* to enhance its survival under harsh acid conditions and in yogurt. Fozo et al. (2004) observed that an increased fatty acid length (an increase in the proportion of C19:0 cyclopropane fatty acids and a lower ratio of saturated to unsaturated membrane fatty acids) was an important membrane alteration that increased survival in acidic environments.

2.2.3.3 Growth phase and cell harvesting conditions

Bacterial growth occurs in four distinct phases, i.e., lag, log, stationary, and death phases. The stress response of the bacterial culture is dependent on the growth phase. For instance, bacteria that enter into the stationary phase develop a general stress resistance.

They are more resistant to various types of stress response than bacteria in the log phase because carbon starvation and exhaustion of available food sources trigger responses that allow the survival of the cell population (Van de Guchte et al. 2002). For example, probiotic spray dried powders containing high numbers of viable cells (over 50% survival; 2.9 x 10^9 CFU/g) were produced when stationary phase cells of *Lactobacillus rhamnosus* were used, while early log phase cultures exhibited only 14% survival, (2.1x10^7 CFU/g) survival (Corcoran et al. 2004). In a similar study, *L. delbrueckii* subsp. *bulgaricus* NCFB1489 cells harvested from stationary phase, showed higher survival compared to the compared with exponential phase harvested cells (Teixeria et al. 1996). In summary, appropriate growth phase and cell harvesting conditions are essential for the stabilization of bacteria.

2.2.3.4 Growth media and growth conditions

Lactobacillus spp. are often grown in a De Man, Rogosa, and Sharpe (MRS) medium, which is a selective medium for the growth of lactobacilli. However, the presence of sodium acetate lowers the pH of MRS medium to 5.7 this lower pH inhibits the growth of some gram negative bacteria, but still allows the growth of most lactobacilli spp. Bâati et al. (2000) observed that variation in the environmental conditions of the growth medium resulted in alteration in the fluidity of the cytoplasmic membrane. Membrane fluidity is responsible for regulation of the flow of nutrients and metabolic products into and out of the cell (Annous et al. 1999). For example, when lactic acid bacteria were grown in the presence of Tween 80, there was an increase in their resistance to freeze drying because of a change in the lipid composition of the membrane (Goldberg and Eschar, 1977) and there was an improvement in the bile salt tolerance (Kimoto et al. 2002). Silva et al. (2005) showed that the cells of *Lactobacillus delbrueckii* subsp. *bulgaricus*, when grown under controlled pH conditions, were more sensitive to the stress-

es encountered during starter culture production/storage and handling. In contrast, the noncontrolled cells showed better viability because of enhanced production of heat-shock proteins. These results indicate the importance of growth media and cell harvesting conditions in the stabilization of probiotic bacteria.

2.2.4 Stabilization by optimizing the storage conditions

Long term storage of lactic acid bacteria for use in various products is a common practice in the food industry. It is important to mention factors such as temperature, relative humidity, oxygen content, type of probiotic carrier, and the packaging material during storage. These factors alone or in combination can lead to loss of viability as a result of deteriorative chemical reactions. Overall, optimization of the storage conditions could possibly improve the survival rate of bacteria during storage.

Temperature is an important parameter that influences the viability of probiotic bacteria during storage. Storage at high temperature results in poorer survival rates than storage at lower temperatures (Gardiner et al. 2000; Simpson et al. 2005). For extended periods, storage at −18ºC has been found to be more suitable for freeze dried probiotic bacteria than storage at 20ºC (Bruno and Shah, 2003). The loss of viability for *Bifidobacterium* spp. has been found to be lower with storage at 4ºC (2%) than with storage at 25ºC (40%) for 90 days. Increased survival at low temperature has been associated with a lower rate of fat oxidation (Gunning et al. 1995).

Relative humidity is another variable that controls the viability of probiotic bacteria. During storage, a desiccant enhanced the viability of *Bifidobacterium longum* B6 and *Bifidobacterium infantis* CCRC 14633 by removing moisture (Hsiao et al. 2004). Gunning et al. (1995) observed that a relative humidity of 11% was the optimum to sustain the viability of *Lactobacillus bulgaricus* during storage and that a higher relative hu-

midity resulted in an increased rate of lipid oxidation. During storage at high relative vapor pressure, the crystallization of disaccharides resulted in the loss of viability of freeze dried *Lactobacillus rhamnosus* GG. The presence of oxygen is detrimental to the survival of bacteria because oxidative damage occurs during long term storage. Oxygen radicals can damage the polyunsaturated fatty acids present in the phospholipids of the bacterial cell membrane (Halliwell et al. 1993). Oxygen-depleting agents, such as oxygen absorbers and antioxidants, improve the viability of the bacteria by providing a low oxygen environment and preventing membrane lipid degradation. Nitrogen flushing has been found to improve bacterial viability (Gunning et al. 1995) and vacuum packaging is another alternative technique for the removal of oxygen. Jin et al. (1985) advocated that low oxygen levels are important for the stabilization of bacteria during long term storage.

Amorphous carbohydrates in probiotic carriers play an important role in storage stability by impeding the detrimental process. They exist as noncrystalline solids (glass) below their glass transition temperature (Miao et al. 2008). Importantly, the formation of a glassy state in a probiotic carrier limits membrane lipid oxidation, protein unfolding, and chemical degradative reactions by providing an effective environmental barrier and, thereby, minimizing the transitional molecular motion (Sun and Leopold 1997; Crowe et al. 1998). The effect of the glassy state on the survival of probiotic bacteria has been studied in spray drying (Ananta et al. 2005) and freeze drying (Miao et al. 2008). Ananta et al. (2005) spray dried *Lactobacillus rhamnosus* GG in three spraying media, i.e., reconstituted skim milk (RSM), RSM with Raftilose 95, and polydextrose. The survival was greater in RSM than in the other media for storage at 37ºC and 25ºC at 11% relative vapor pressure for 4 weeks. Miao et al. (2008) studied the viability of freeze dried *Lactobacillus paracasei* and *L. rhamnosus* GG on storage at 22−25°C for 38−40 days at

different relative humidities. They also noted the effect of a protective medium consisting of RSM or a cryoprotective disaccharide, i.e., lactose, trehalose, sucrose, maltose, and a mixture of disaccharides. Physical changes, such as collapse, crystallization, and stickiness, have been associated with higher relative vapor pressure. A significant loss in viability above a relative vapor pressure of 11.4% was observed and it was concluded that the difference between the storage temperature and the glass transition temperature (T − Tg) influenced the viability.

Packaging materials play an important role in the storage stability of bacteria by providing excellent barriers against gas and water vapor. Hsiao et al. (2004) showed that microencapsulated *Bifidobacterium* cells had better viability when stored in glass bottles than when stored in PET bottles, possibly because of the higher oxygen barrier properties of glass bottles than of PET bottles. As aluminum provides a barrier to both oxygen and moisture, which are injurious to bacteria during long term storage, a significant improvement in the viability of *Bifidobacterium* spp. was seen during storage in polythene bags within aluminum-coated paper bags (Simpson et al. 2005). Polyester−aluminum−polythene laminate, which has gas and water vapor impermeable properties, provides a superior barrier for stabilizing bacteria (Jin et al. 1985).

Storage time has an inverse relationship with bacterial viability. During storage, there is a change in the ratio of linoleic/palmitic acid (C18:2/C16:0) or linolenic/palmitic acid (C18:3/C16:0), which is linked to the viability of freeze dried bacteria (Yao et al. 2008; Coulibaly et al. 2009). Other chemical damage mechanisms include the formation of lipid hydroperoxides from free radical reactions, which decompose to secondary products, such as malonic dialdehyde (Raharjo and Sofos, 1993). Simpson et al. (2005) has

shown that there is a significant loss in the viability (greater than 4 log reduction) in spray dried Bifidobacterium spp. during storage for a period of 3 months at 25°C.

2.3 Knowledge gaps

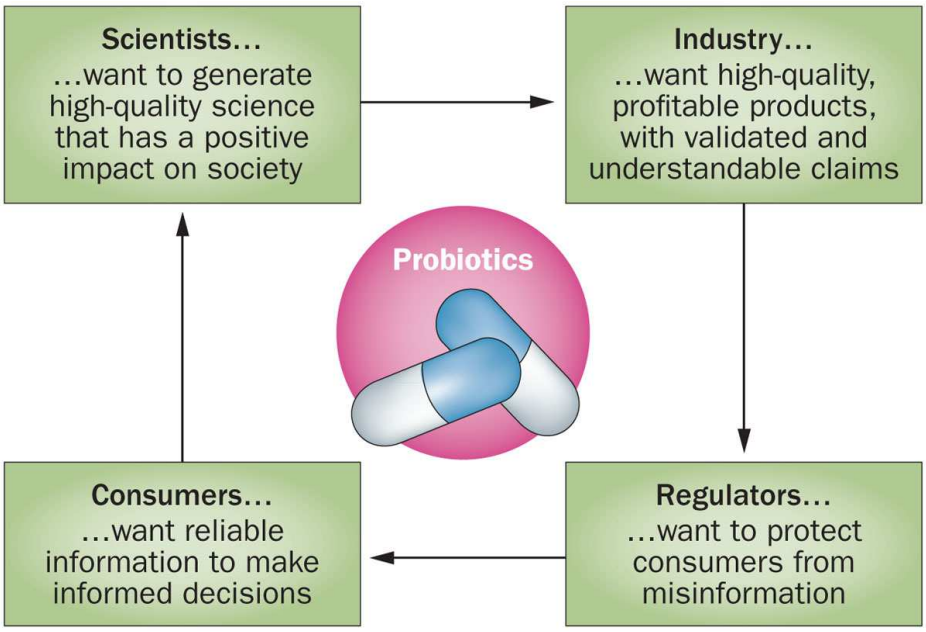

Figure 2-2 Objectives of stakeholders in the field of probiotics (Hill et al. 2014)

The association between the probiotic stakeholders has been outlined in Figure 2-2. Researchers over the years have collected wealth of information in relation to the health benefits associated with the consumption of probiotics. However, industry is unable to take the goodness of probiotics to the wider public due to the lack of potential stabilization strategy to apply probiotics in ambient shelf-stable food products. In the present scenario, probiotic micoorganisms are being mainly delivered through refrigerated short shelf-life products. Even if incorporated into ambient shelf-life products, the products generally fail to meet the regulatory criteria, which require probiotic bacteria to be viable in high numbers at the end of shelf-life period.

From the present literature review, it is established that researchers have tried a flurry of techniques to improve the viability of probiotic bacteria during storage. The key technologies involve:

1. Stabilization using drying or desiccation, such as freeze drying, spray drying, fluidized bed drying and vacuum drying or mixed/ two-step drying process (Ananta et al. 2005; Simpson et al. 2005; Santivarangkna et al. 2007).

2. Encapsulation processes, such as emulsion techniques, extrusion techniques and coacervation (Hsiao et al. 2004; Mokarram et al. 2009).

3. Stabilization by manipulating the cell physiology, stress adaptation by heat-stress, osmotic stress, acid stress, optimization of growth phase, cell harvesting conditions, growth media and growth conditions (Van de Guchte et al. 2002; Prasad et al. 2003; Corcoran et al. 2004).

4. Optimization of storage and packaging conditions. (Hsiao et al. 2004).

Drying processes over the years have gained major attraction for application into shelf stable food products. However, it has been observed that all the drying processes, used to embed bacteria result in some loss of viability, during dying and subsequent storage. In freeze drying, this is due to ice crystal formation and rupture of the cell membrane (Fonseca et al. 2004). In spray drying, the high temperature results in the thermal inactivation of the cells (Ananta et al. 2005; Fonseca et al. 2000; Teixeira et al. 1995). In contrast, fluidized bed drying uses mild temperatures and therefore minimises bacterial inactivation (Bayrock and Ingledew, 1997). Fluidized bed drying has been widely used in the production of wine yeast, but has found limited application in the production of concentrated viable bacteria (Caron, 1995). However, a recent study showed that fluidized

bed drying of the bacterial cells in a dairy matrix, improved the stability of probiotic *Lactobacillus* sp., with a viable cell count of 100 million cells per gram of dried probiotic powder after 52 weeks of storage at 25˚C (Nag and Das, 2011). Therefore, the aim of the thesis is to fill the knowledge gaps to develop shelf-stable probiotics by gaining understanding on how the matrix structure and bacterial cell physiology manipulation influence the viability of probiotics. Chapter 4 and 5 focus on understanding the importance of matrix component, matrix structure in relation to improving bacterial viability. Thereafter, the experiments explore the cell physiology manipulation to improve bacterial viability.

Chapter 3 Effect of milk fat, protein and carbohydrate on the viability of probiotic *Lactobacillus paracasei* during storage

3.1 Introduction

The understanding of health benefits associated with the consumption of probiotics has raised interest among consumers, scientific and medical community to apply the therapeutic benefits of probiotics. Probiotic microorganisms could either be added to refrigerated products having low shelf life of few weeks (e.g. yoghurt) or to non-refrigerated dry food products Infant formula or malted beverage. One of the major factors limiting the widespread application is the loss of viability during the shelf life of food products (Iyer and Kailasapathy, 2005; Goderska and Czarnecki, 2008). To claim food as "probiotic", there must be proof of delivery of viable strain(s) at an efficacious dose at the end of shelf-life (Hill et al. 2014). Although, immobilization of bacteria via drying technology in a protective matrix remains the most common means to stabilize bacteria, limited knowledge is available on retaining the viability of probiotics in dry ambient long shelf life products. The main objective of immobilization or encapsulation in a protective matrix is to create a microenvironment that protects probiotics from harsh external conditions (Anal and Singh, 2007). The immobilization of probiotic bacteria in a matrix capable of minimizing loss of viability during storage at elevated temperatures provides a potential route for incorporation of probiotics in dry food products.

Commercially, there is a demand for the development of concentrated viable probiotics for use in non-refrigerated food products. Potentially, both freeze drying and spray drying can be used for the production of concentrated probiotic powders, but both these technologies use adverse temperature conditions that are injurious to the bacteria (Gibbs et al. 1999; Kailasapathy, 2002; Fowler and Toner, 2005; Madene et al. 2006). Fluid-

ized bed drying on the other hand is a potential drying technique where the bacteria do not attain the temperature of air as a result of evaporative cooling. It has been widely used for the production of baker's yeast and wine yeast but to date have found limited applications for the production of concentrated viable bacteria (Caron, 1995). However, a recent study showed that fluidized bed drying of the bacterial cells in a protective dairy matrix improved the stability of probiotic *Lactobacillus* sp. with a viable cell count of 100 million cells per gram of dried probiotic powder after 52 weeks of storage at 25 ˚C (Nag and Das, 2011). It was therefore of interest to study how the individual matrix components affected the bacterial viability. The composition and structure of matrix within which the probiotic bacteria are contained are important for the stability of the bacteria. Carbohydrates, protein and fat all play protective role for the bacteria. Carbohydrates, such as lactose, can be used in the drying matrix to protect the cells against drying stress, and to substitute for the hydrogen bonded water in the head group of the phospholipid bilayers present in the bacterial cell membrane (Sun and Leopold, 1997; Crowe et al. 1998; Miao et al. 2008; Strasser et al. 2009). In addition, the formation of glassy (amorphous) state during drying can impart very high viscosity, which can act as a protective encapsulation for the bacteria, limiting water and oxygen exchange (Sun and Leopold, 1997; Crowe et al. 1998). However, during storage at high water activity conditions, the crystallization of carbohydrate leads to bacterial death (Miao et al. 2008; Strasser et al. 2009). Proteins can delay the crystallization of carbohydrates to maintain the protective glassy state, by reducing the rate of diffusion of sugar molecules to form crystals (Haque and Roos, 2004; Jouppila and Roos, 1994; Shrestha et al. 2007). Similarly, fat may also delay the crystallization of lactose by reducing the water absorbing properties due to the increased surface hydrophobicity (Knudsen et al. 2002; Kim et al. 2003; Lahtinen et al. 2007). Therefore, for this prelimi-

nary study, fluidized bed drying of bacteria in three dairy matrices, i.e. whole milk powder (WMP), skim milk powder (SMP) and milk protein isolate (MPI) was carried out. WMP represents a matrix rich in fat, protein and lactose, SMP represents a matrix rich in protein and lactose and devoid of fat, while MPI represents a matrix rich in protein, without fat and lactose. The examination of bacterial viability during storage in these matrices with specific compositions was thought to provide a base for further experiments and a better understanding towards the importance of matrix constituents.

The experiment was designed to test the effects of WMP, SMP or MPI on the bacterial viability loss during storage, based the storage water activity of the powders. The powders were taken out at specific intervals during the fluidized bed drying process, to obtain water activity a_w levels of 0.3, 0.4 and 0.5, followed by vacuum packing in aluminium foil pouches. The vacuum packaging of the powders, in the aluminium foil, provided water vapour barrier protection, thereby maintaining the water activity levels during storage. The powders were further subjected to variety of storage temperatures (4, 25 and 37 ºC).

3.2 Materials and methods

Whole milk powder (WMP), skim milk powder (SMP) and milk protein isolate (MPI) were obtained from Fonterra Co-operative Group Ltd, New Zealand.

3.2.1 Enumeration of viable bacteria

deMan, Rogosa and Sharpe (MRS) agar was used to enumerate viable *L. paracasei* 431 present in the powder samples that were stored for a period of up to 4 weeks. The samples were homogenized in sterile buffered peptone water (5 g/l Merck, Germany) for 5 min using a Stomacher 400 Lab Blender (Seward Medical, London, UK). From

this homogenate, decimal serial dilutions were made in the same sterile peptone water and used for microbiological analyses. For determination of viable cells, diluted samples were pour plated on MRS agar plates (Merck), after 10 fold serial dilution in peptone water. After solidification of the agar, individual bacterial cells are fixed, which allows them to multiply during incubation and form colonies. The visible colonies developed after 24-48h incubation; viable cell counts were determined after 48 hours incubation under aerobic conditions at 37ºC. Colonies counted were then multiplied with the dilution factor to obtain total viable cell counts and recorded as colony forming units (CFU) per gram of product. Three batches of the sample powders were made and analysed for viability.

3.2.2 Bacterial growth and cell harvesting conditions

Lactobacillus paracasei subsp. *paracasei* CRL 431 (ATCC 55544) was grown batch-wise in a 5 L durham bottle in 55 gL^{-1} MRS medium (Merck KGaA, Darmstadt, Germany) at 37 ºC. The fermentation took place under microaerophilic conditions. Bacterial growth curve was obtained by observing the viability counts during the growth cycle, as outlined in section 3.2.1 i.e. Enumeration of viable bacteria.

The cells in the stationary phase (18 h after inoculation) were harvested by centrifugation (10 min at 15000g, in a Sigma 6-16S centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The stationary phase cells were used in the current study, based on previous research finding (Corcoran et al. 2004), indicating that stationary phase cells provided better viability during storage .

The harvested cells were washed in buffered peptone water (5 gL^{-1}) (Merck), resulting in a cell pellet, containing ~ 3 x 10^{11} CFU/g of bacterial cells.

3.2.3 Fluidized bed drying

The harvested cells were mixed manually with WMP, SMP or MPI for 10 min. The matrix−bacteria mixture was dried in a laboratory fluidized bed drier (model Uni-Glatt, Glatt GmbH, Binzen, Germany) with dehumidified compressed air at 40 ºC. In case of fluidized bed drying of bacterial powders, the inlet air temperature is carefully chosen so as to maintain a delicate balance between the moisture evaporation from the granulated surface and the migration of moisture through the capillaries from the interior of the granulate to the surface, as dried. If the inlet air temperature is too high, a surface crust formation is generally seen which will prevent the moisture removal from deeper layers to the outside. The crust formation delays the drying process with the increased amount of moisture remaining within the crust, leading to increased viability loss during storage.

In the fluidized bed drier, air travels upward through the bed of particles with sufficient velocity to provide fluid like behavior, and the freely suspended particles in the air stream are dried by rapid heat exchange and mass transfer (Bayrock and Ingledew, 1997) (Figure 3.1). The Uni-Glatt laboratory fluid bed dryer has a batch size capacity of 1 to 1.5kg (Figure 3.2).

The powders were taken out at various stages during the drying process to achieve powders with final water activity levels of 0.3, 0.4 and 0.5. Fluidized bed drying used in the present study aided in rapid drying of the powder-bacteria mixture.

3.2.4 Packaging and storage

Powders produced were immediately vacuum-packed in aluminium foil and stored at three different temperatures; 4ºC, 25ºC and 37ºC. Vacuum packaging in aluminium foil

was done due to its best water vapour barrier properties (Jin et al. 1985; Simpson et al. 2005), which may help in maintaining the water activity of the powders during storage.

The influence of the storage temperature and water activity (a_w) on the viability of the embedded bacteria was studied for a period of up to 4 weeks.

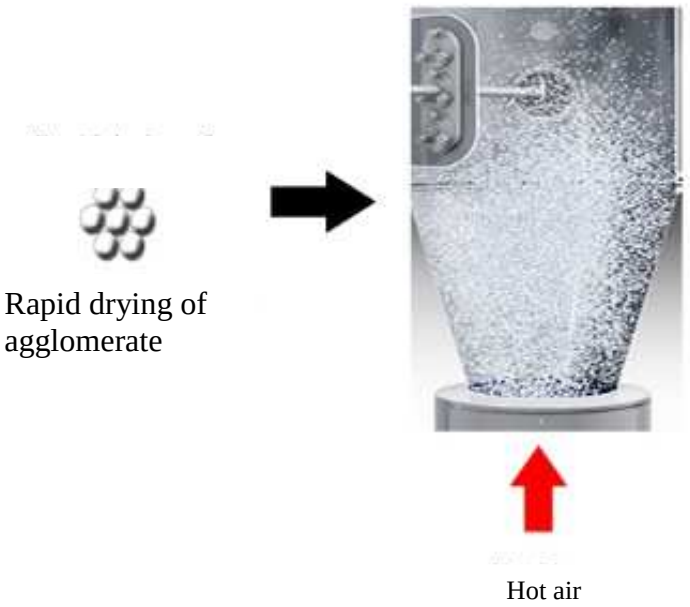

Figure 3-1 Schematic representation of fluidized bed drying process

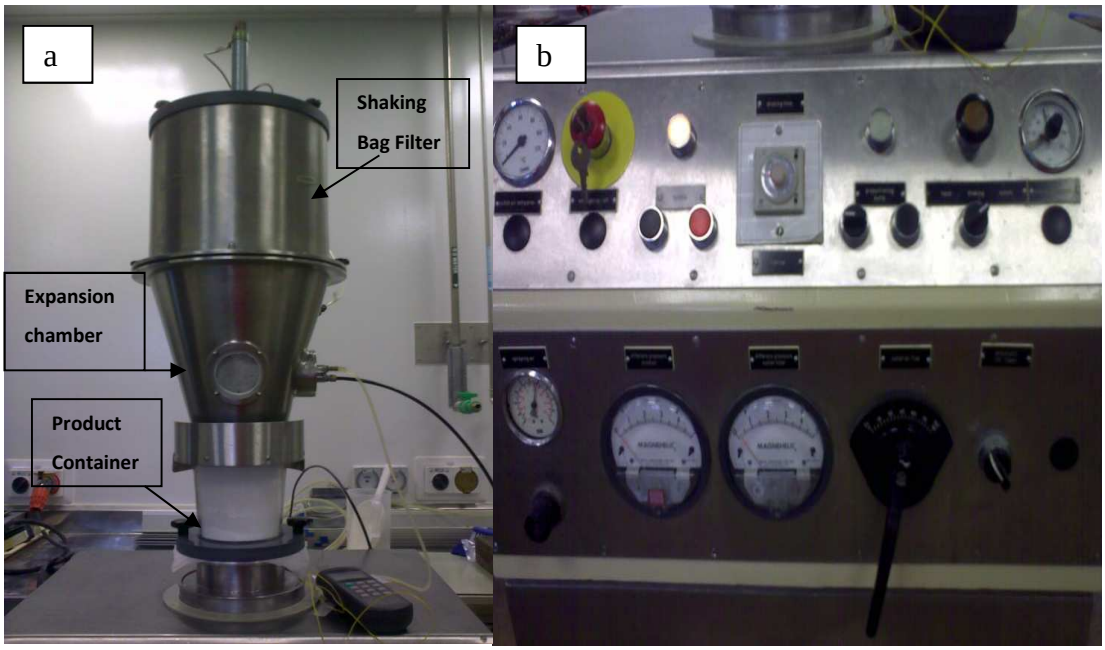

Figure 3-2 (a) Uni-Glatt Laboratory scale fluid bed dryer and (b) control panel

3.2.5 Water activity measurement

Water activity represents the energy level of water in a product. Water activity is defined as the ratio of the vapor pressure of water in a sample to the vapour pressure of pure water at the sample temperature. *Lactobacillus paracasei* embedded in protective matrix was periodically taken out during drying to have samples with water activity (a_w) of 0.3, 0.4 or 0.5 (±0.01). Water activity was measured using Decagon CX-2 Water Activity (a_w) Instrument (Decagon Devices Inc., WA, USA) at 25°C.

3.2.6 Confocal laser scanning microscopy

To study the location of the bacteria within the matrix, confocal laser scanning microscopy was employed. The idea was to locate, live and dead bacteria within the matrix. Confocal laser scanning microscope basically scans a sample sequentially point by point, line by line or multiple points at once and assembles the pixel information to one image. As a result, optical slices of the specimen are imaged with high contrast and high resolution in x, y and z planes. The image stacks can be combined to create 3D (dimensional) view.

Acridine orange (sigma), propidium iodide (PI) (Sigma), nile blue (Sigma) and fast green (Sigma) were the stains used in this study. The cells stained with PI were observed not to take up acridine orange. Researchers had previously reported that the dead cells are not stained by acridine orange (Roszak and Colwell, 1987). PI is not membrane impermeable and hence is generally excluded from live cells. In case of compromised cell membrane, PI binds with the GC (guanine-cytosine) rich region of DNA and causes dead cells to fluoresce (Swope and Flickinger, 1996). Nile blue is a lipophilic stain which reacts with the milk fat to generate fluorescence (McKenna,

1997). Fast green in this case has been used to create contrast from acridine orange stained protein with fast green. All the stains were dissolved in commercially available glycerol based mounting medium Dako (Dako Corporation, Carpinteria, CA 93013, USA) at a concentration of 1 mg per ml (McKenna, 1997). The use of mounting medium as a dye carrier prevented dissolution of the matrix and aided in visualization of intact matrix. The laser intensity was 10% and the images were taken at an increasing depth from the surface with increments of 0.5µm. The list of the dyes used in the study is shown in Table 3-1.

Table 3-1 List of dyes and excitation emission filters used in this study

Dye	Excitation/ Bandpass filter	Function	Reference
Acridine Orange	488/ 500-540	To stain live, recoverable, growth responsive, metabolically active, dormant and active cells	(Roszak and Colwell, 1987)
Propidium iodide	488/550-620	To stain dead cells	(Swope and Flickin-ger, 1996)
Nile blue	488/550-620	To stain fat	(McKenna, 1997)
Fast Green	633/650-700	To stain protein	(Auty et al. 2001)

3.2.7 Scanning electron microscopy

In scanning electron microscopy (SEM), a focused beam of high-energy electrons interacts with the sample to generate a variety of signals at the surface of the solid sample specimen. The information that may be obtained from SEM includes external

morphology (texture), chemical composition, crystalline structure and orientation of the material making up the structure.

Surface topology of the encapsulated bacterial powder was studied using SEM. Dry milk powders were sprinkled onto double-sided tape on an aluminium SEM specimen stub, the loose particles blown off with a hand air 'puffer', the samples sputter coated with gold and viewed in a FEI Quanta 200 Scanning Electron Microscope. Digital images were saved at the required magnifications.

3.3 Results

3.3.1 Growth curve of *L. paracasei* subsp. *paracasei* CRL 431

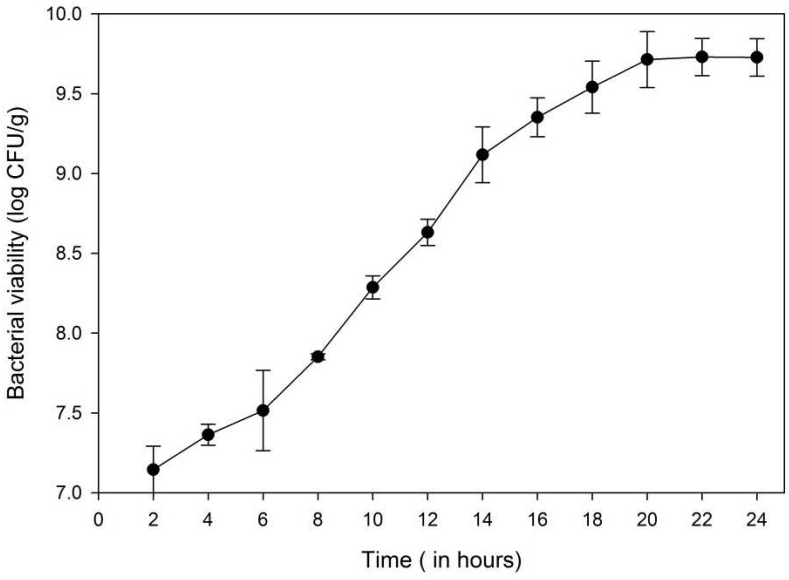

Figure 3-3 Typical growth curve of *Lactobacillus paracasei* subsp *paracasei* CRL 431 over a 24hr period at 37ºC under microaerophilic conditions in MRS medium.

The growth curve of *L. paracasei* subsp. *paracasei* CRL 431 is shown in Figure 3-3. The bacteria reached stationary phase after 18h.

3.3.2 Bacterial viability

The study evaluated the effect of matrix, powder water activity during storage and storage temperature on the viability of the probiotic bacteria. *L. paracasei* was embedded in three dairy matrices, WMP, SMP and MPI using fluidized bed drying. The embedded bacteria in were taken out at specific intervals during the drying process to achieve final a_w of powder as 0.5, 0.4 or 0.3. The powders were subsequently vacuum packed in aluminum foil to maintain the required a_w. The powders were stored at three different temperatures; 4ºC, 25ºC and 37ºC, for a period of up to 4 weeks.

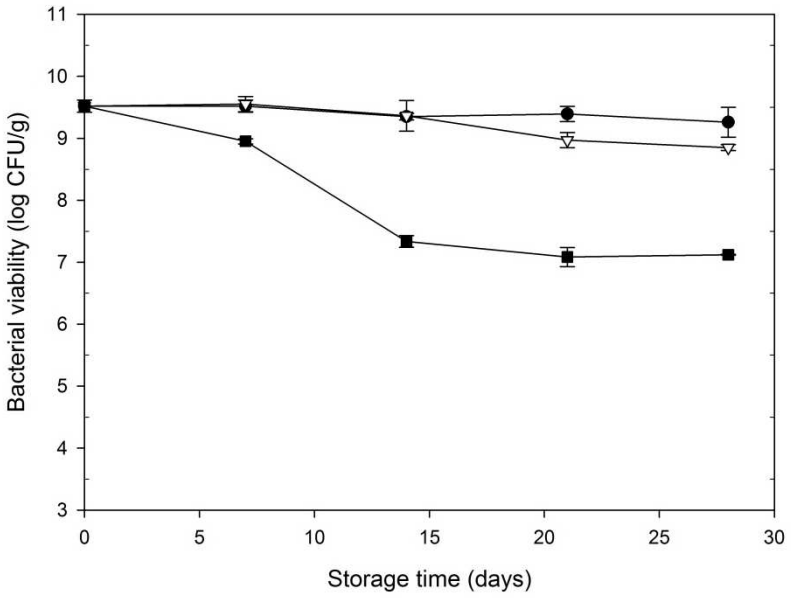

Figure 3-4 The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in whole milk powder having 0.3 a_w. The viability is expressed as the logarithmic values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3)

The bacteria embedded in WMP matrix having 0.3 a_w (Figure 3-4) had an initial viability count of 9.51±0.09 log CFU/g. During storage period of 4 weeks considerable

loss in bacterial viability was observed, the sample stored at 37ºC showed the largest loss, where an initial decline to 7.33 ±0.09 log CFU/g was observed during the 14 day period. However, during the next 14 day period the viability trend remained constant, and the bacteria viability at the end of storage period was 7.12±0.01 log CFU/g. A mild decline in viability was observed in the samples stored at 25ºC, with 8.95±0.04 log CFU/ g of viable bacteria remaining at the end of storage period. Storage at 4ºC resulted in better bacterial viability (9.28±0.21 log CFU/g at the end of storage period), as compared to the bacterial samples stored at 25 and 37ºC.

The bacteria embedded in SMP matrix with 0.3 a_w (Figure 3-5) had an initial viability count of 9.75±0.14 log CFU/g. During the storage period of 4 weeks considerable loss in bacterial viability was observed, the sample stored at 37ºC, showed the largest loss where an initial decline till 7.64±0.11 log CFU/g was observed during the first 14 day period. However, there were abrupt loss in viability thereafter and less than 3.0 log CFU/g was observed during subsequent viability check. A mild decline in viability was observed in the samples stored at 25ºC, with 8.34±0.06 log CFU/g of viable bacteria remaining at the end of storage period. Storage at 4ºC resulted in retaining better bacterial viability (9.6±0.07 log CFU/g at the end of storage period), as compared to the bacterial samples stored at higher temperature 25 and 37ºC respectively.

The bacteria in MPI matrix with 0.3 a_w (Figure 3-6) had an initial viability count of 9.75±0.15 log CFU/g. During the storage period of 4 weeks considerable loss in bacterial viability was observed, the samples stored at 37ºC showed the largest loss where an initial decline till 5.35±0.18 log CFU/g was observed during the first 14 day period. However, during the next 14 day period the viability trend remained constant, and the bacteria viability at the end of storage period was 4.59±0.10 log CFU/g. A slight

decline in viability was observed in the samples stored at 25ºC, with 8.24±0.12 log CFU/g of viable bacteria remaining at the end of storage period. Storage at 4ºC resulted in better bacterial viability (9.68±0.18 log CFU/g at the end of storage period) as compared to the bacterial samples stored at higher temperature 25 and 37ºC respectively.

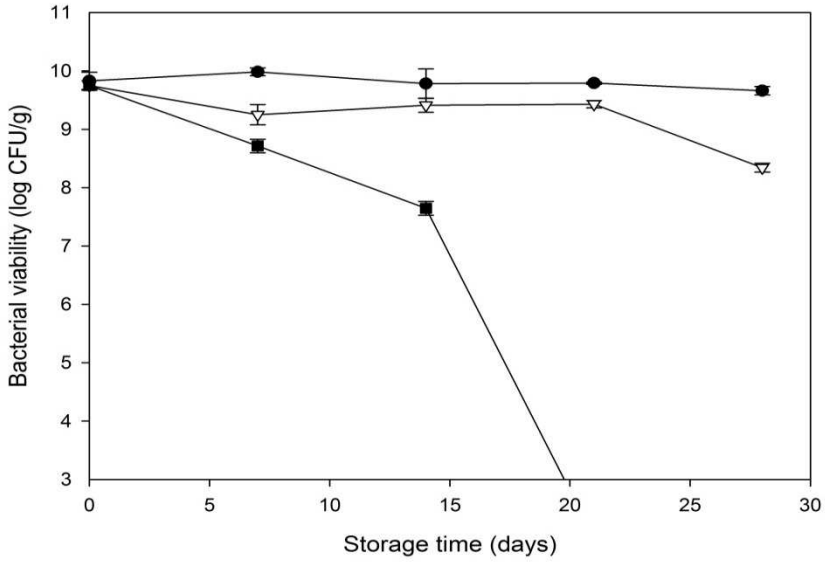

Figure 3-5 The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in skim milk powder having 0.3 a_w. The viability is expressed as the logarithmic values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3)

The bacteria in WMP matrix with 0.4 a_w (Figure 3-7) had an initial viability count of 9.46±0.15 log CFU/g. During the storage period of 4 weeks considerable loss in bacterial viability was observed, the samples stored at 37ºC showed the largest loss where an initial decline till 7.43±0.11 log CFU/g was observed during the first 14 day period. However, during the next 14 day period there was an abrupt decline and less than 3.0 CFU/g was observed, till the end of the storage period. The samples stored at 25ºC and 4ºC had a viable count of 8.99±0.16 and 9.12±0.02 respectively.

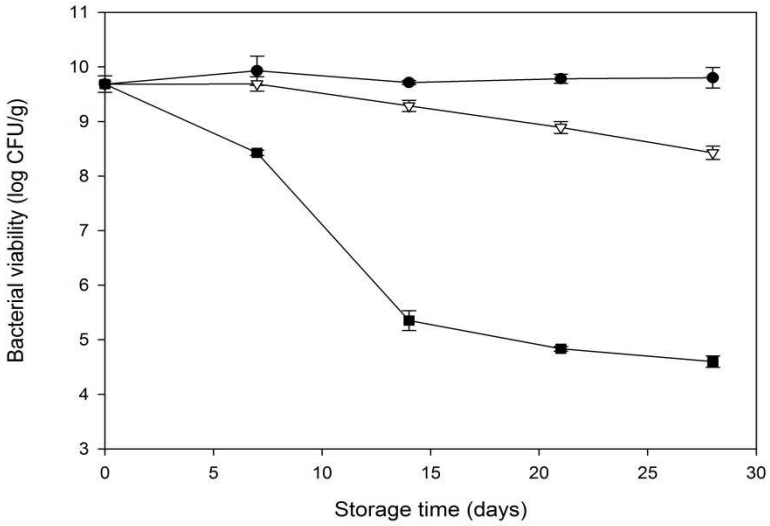

Figure 3-6 The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in milk protein isolate having 0.3 a_w. The viability is expressed as the logarithmic values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3)

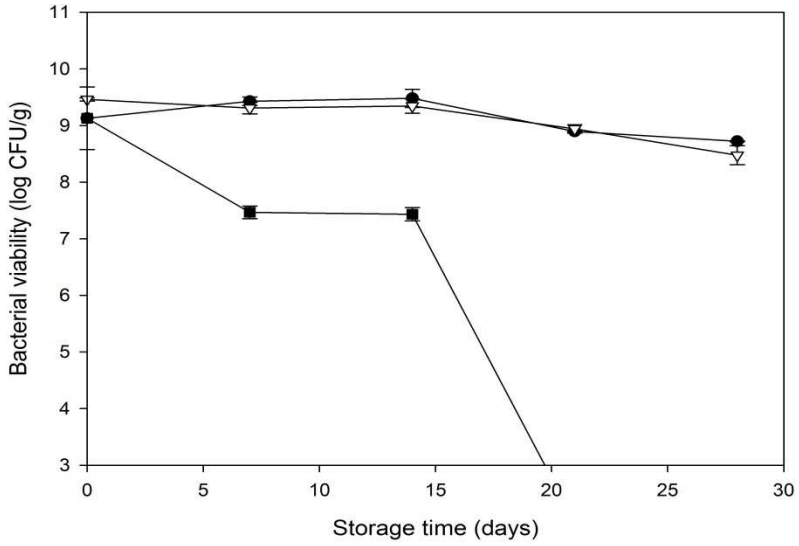

Figure 3-7 The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in whole milk powder having 0.4 a_w. The viability is expressed as the logarithmic values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3)

The bacteria in the SMP matrix with 0.4 a_w (Figure 3-8) had an initial viability count of 9.90±0.19 log CFU/g. In case of the bacteria stored at 37ºC, rapid drop in the viable cell count of 7.35±0.17 log CFU/g was observed during the first 14 day period. Thereafter, less than 3.0 CFU/g was observed, till the end of the storage period. The sample stored at 25ºC and 4ºC resulted in better viability result, 8.93±0.08 and 9.89±0.06 log CFU/g at the end of storage period.

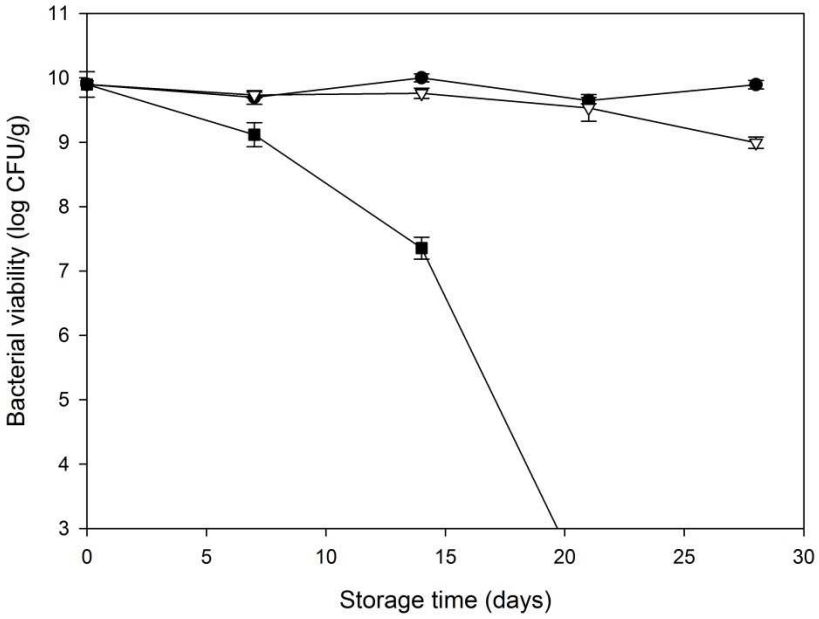

Figure 3-8 The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in skim milk powder having 0.4 a_w. The viability is expressed as the logarithmic values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3).

The bacteria encapsulated in MPI matrix having 0.4 a_w (Figure 3-9) had an initial viability count of 9.93±0.12 log CFU/g. Storage at 37ºC resulted in total loss in the bacterial viability within a week of storage. The sample stored at 25ºC had a viability count of 5.72±0.14 log CFU/g at the end of storage period while the bacteria stored at 4ºC had a viability count of 9.37±0.29 at the end of storage period.

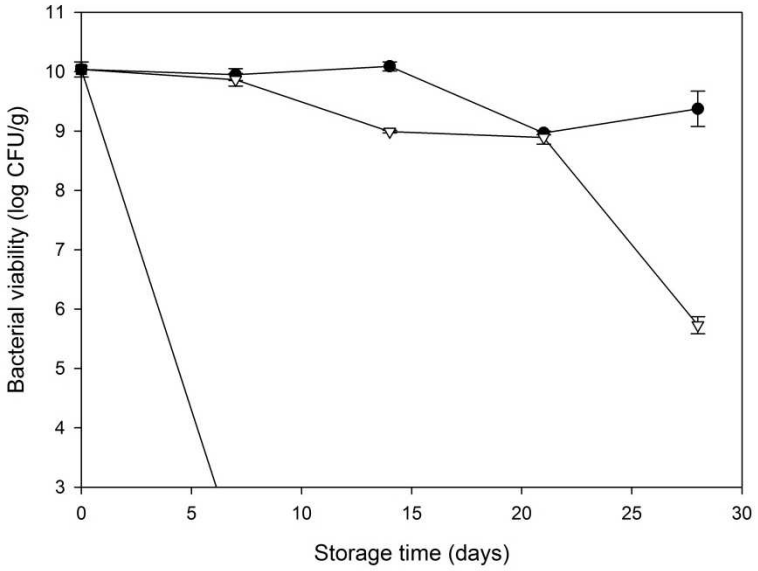

Figure 3-9 The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in milk protein isolate having 0.4 a_w. The viability is expressed as the logarithmic values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3)

The bacteria encapsulated in WMP, SMP MPI matrix having 0.5 a_w had an initial viability count of 9.73±0.09 log CFU/g, 9.44±0.14 log CFU/g and 9.44±0.15 log CFU/g respectively. Storage at 37ºC resulted in total loss in the bacterial viability within a week of storage. Storage at 4 and 25ºC resulted in complete viability loss at the end of storage period (refer appendix A4).

3.3.3 Morphology of powders

Scanning electron microscopy images of the *L. paracasei* embedded in WMP, SMP and MPI are shown in Figure 3-10. There were no visible bacteria on the surface of the powders suggesting sufficient embedding of the bacteria within the matrix. The MPI matrix (Figure 3-10 a & b) had a shiny lustrous texture and less rigid structure as compared to SMP matrix (Figure 3-10 c & d) and WMP (Figure 3-10 e & f) matrix.

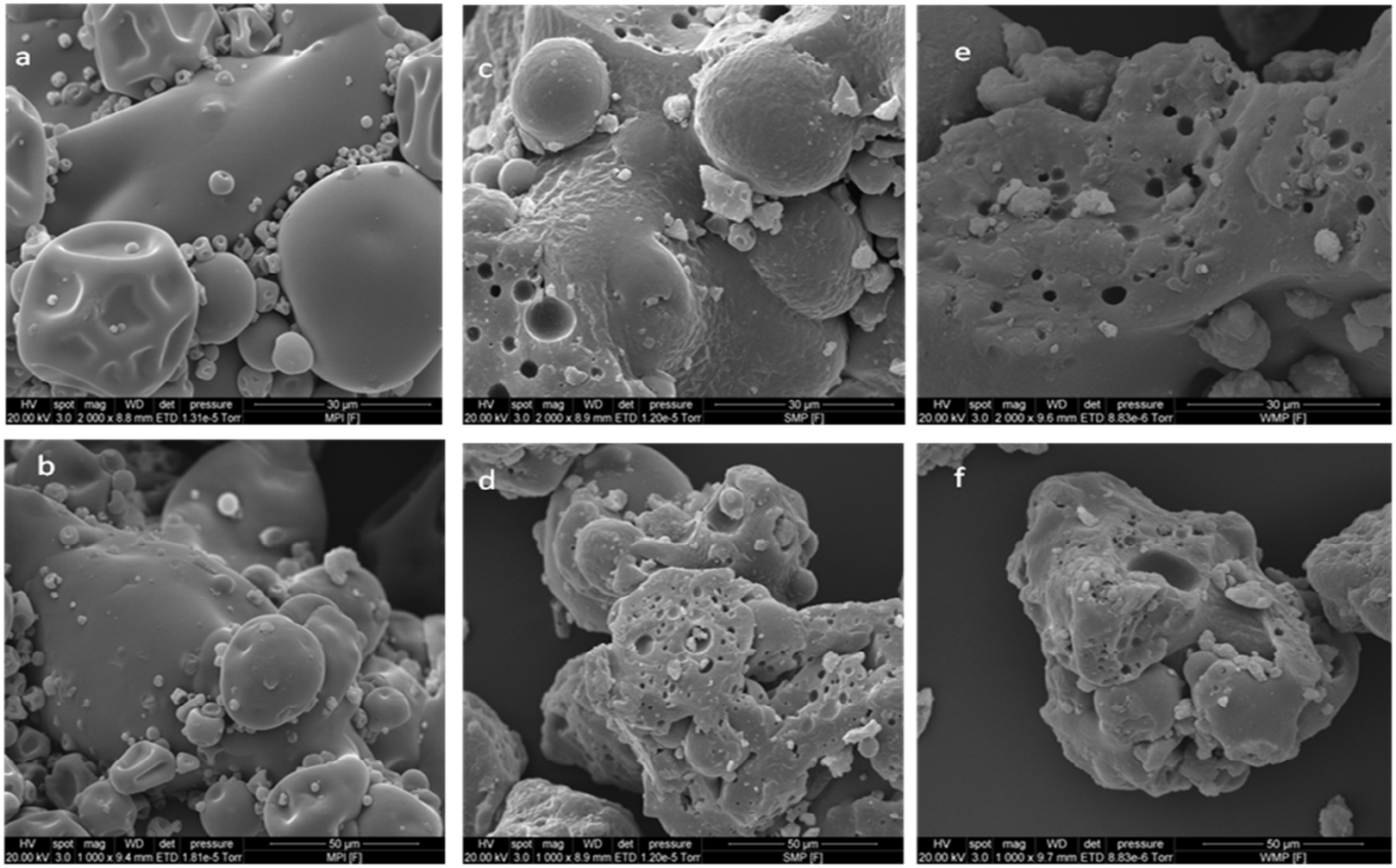

Figure 3-10 SEM images of fluidized bed dried *Lactobacillus paracasei* powders in MPI (a, b), SMP (c, d) or WMP (e, f).

Both SMP matrix and WMP matrix had a similar appearance due to the presence of large amount of lactose, which provides bulk rigidity to the structure (Figure 3-10). Upon storage at higher water activity (0.5 a_w) crystallization was observed in the matrix containing lactose (Figure 3-11b) while the amorphous (non-crystalline) lactose was observed at 0.3 a_w (Figure 3-11a).

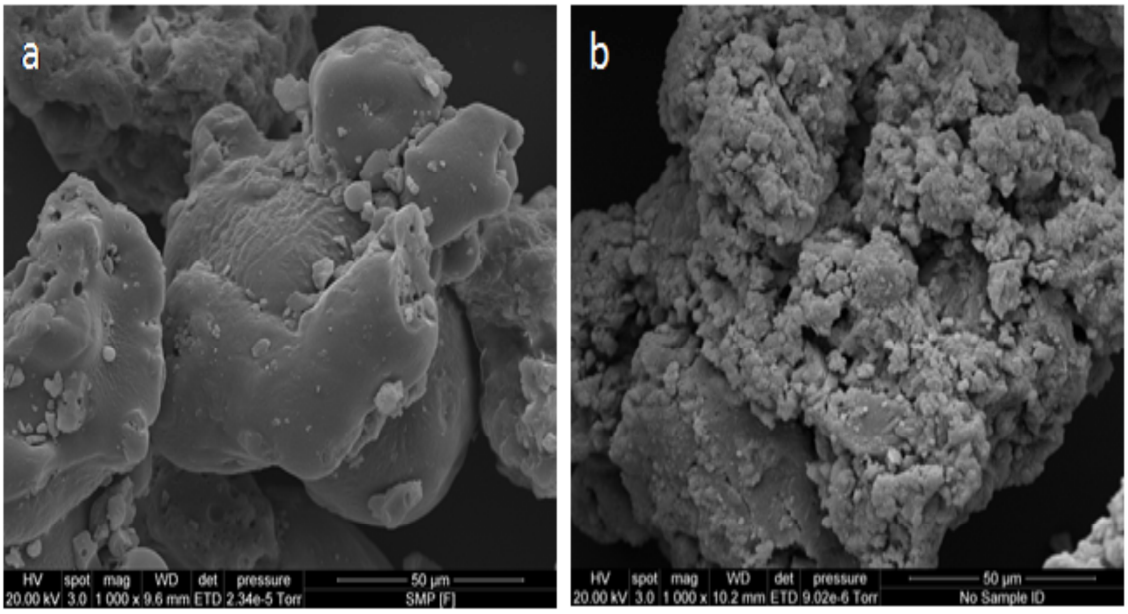

Figure 3-11 SEM image of (a) SMP matrix having 0.3 a_w, b) SMP matrix having 0.5 a_w, upon storage at 25ºC for 28 days.

The Figure 3.11 shows the difference in the powder morphology among the powders after storage at 25ºC. Amorphous glassy structure could be observed in powder with 0.3 a_w (below the critical glass transition temperature) Figure 3-11 a. Crystallization could be observed in the WMP and SMP powders with 0.5 a_w, (above the critical glass transition temperature) Figure 3-11 b.

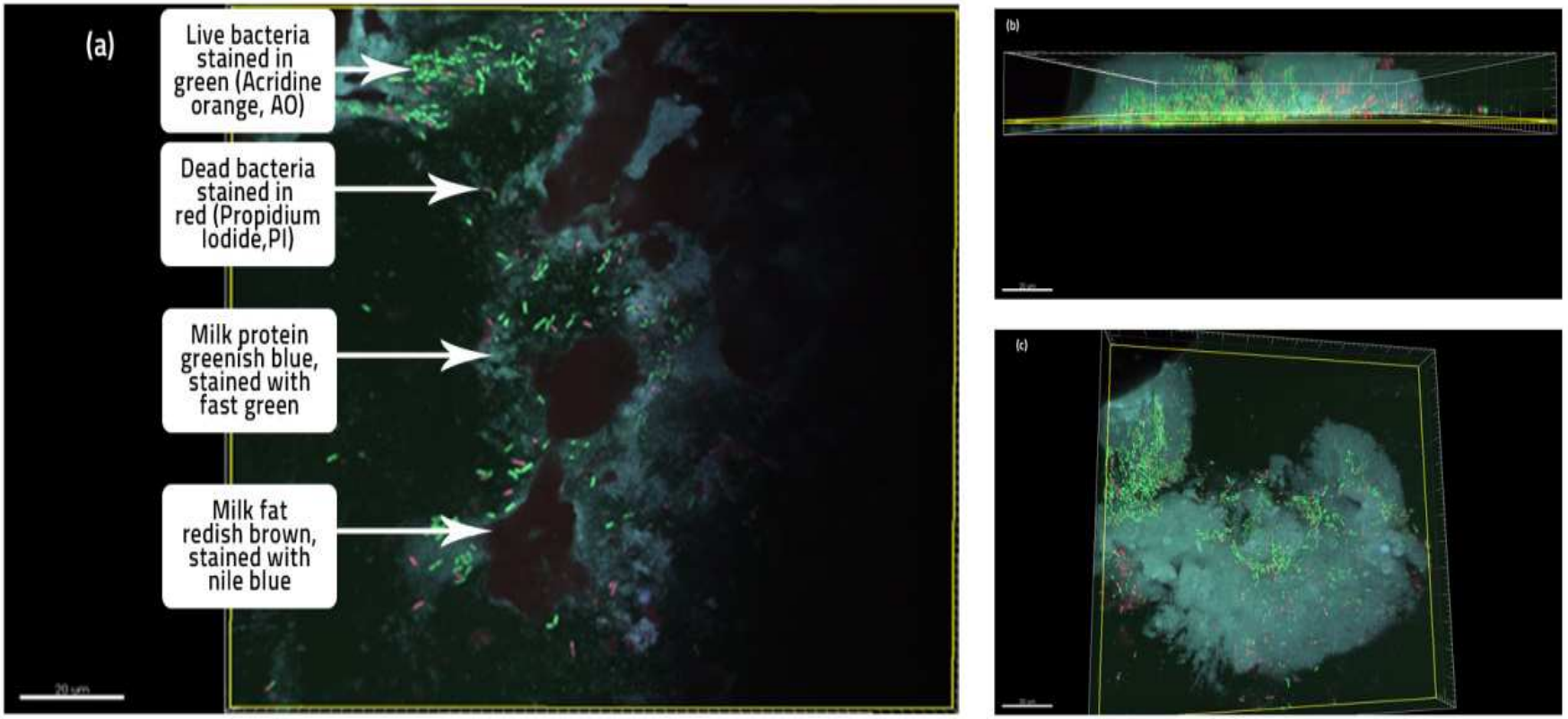

Figure 3-12 CLSM image of (a) surface of WMP matrix, showing immobilization of bacteria within the fat and protein layer (b) cross section of the matrix, (c) spatial distribution of live and dead bacteria which are located below the surface, comprising of bacteria embedded in protein and lactose matrix.

The matrix structure of the bacteria embedded within the whole milk powder was analyzed using confocal laser scanning microscopy (Figure 3-12). The embedded bacteria were observed to lie beneath the surface of the matrix, covered with milk fat. The milk fat could be observed on the surface stained with Nile blue. Milk proteins stained with fast green could be observed to contain the probiotic bacterial mass. Live bacteria were stained with acridine orange and dead bacteria were stained with propedium iodide and could be observed in red.

3.4 Discussion

The aim of the present study was to identify the effects of intrinsic factors, such as drying matrix, water activity and temperature of storage on the viability of the bacteria during storage. Considering the fluidized bed drying process utilizes lower temperature for drying, the thermal inactivation of bacteria during the drying process is markedly reduced. A recent study revealed that fluidized bed drying of the bacterial cells in a protective matrix improved the stability of probiotic *Lactobacillus* sp., during extended storage at 25 ºC (Nag and Das, 2011). Matrix constituents are known to play an important role in providing protection to the bacteria during drying and storage (Miao et al. 2008). Different matrices, WMP, SMP and MPI, were evaluated to provide preliminary understanding on the protective effect of protein (MPI), protein and lactose (SMP) and fat, protein and lactose (WMP) on the bacterial viability during drying and storage. It was hypothesized that the composition of the matrix constituent would affect the survival of bacteria, especially during storage. This is the first study to evaluate the effects of these materials for embedding probiotic bacteria using fluidized bed drying process.

The result demonstrates that higher storage temperature and water activity of the powders leads to increased bacterial death, irrespective of the drying matrices used. The bacterial powders, stored at 4 and 25ºC with a_w, 0.3 and 0.4 had greater bacterial viability compared to the powders with a_w 0.5 and when stored at 37˚C. This finding is consistent with previously published reports, where temperature during storage resulted in lower survival rates of spray and freeze dried *Lactobacillus* paracasei and *Bifidobacterium* sp (Gardiner et al. 2000; Simpson et al. 2005). The loss in viability for *Bifidobacterium* spp. has been found to be lower upon storage at 4ºC than upon storage at 25ºC for 90 days (Bruno and Shah, 2003). Additionally, researchers have observed that increased survival at lower temperature was possibly due to the lower rate of membrane lipid oxidation, thereby preventing cell membrane damage during storage (Gunning et al. 1995). A change in the ratio of linoleic/ palmitic acid (C18:2/C16:0) or linolenic/palmitic acid (C 18:3/C16:0) has also been linked to the viability loss in freeze dried bacteria (Yao et al. 2008; Coulibaly et al. 2009). The formation of lipid hydroperoxides, during storage of bacteria at higher temperature, was also associated with bacterial death upon storage (Raharjo and Sofos, 1993).

The a_w of the stored powders was important for maintaining bacterial viability during storage. With an increase in powder a_w, increased rate of bacterial death occurred, and this was consistent with previous researcher findings (Gunning et al. 1995; Hsiao et al. 2004). Powders were vaccum packaged and stored in aluminium foil pouches, based on earlier reports that the vaccum packaging of probiotic powders in aluminium foil laminates, having high water vapour and oxygen barrier properties, improved the bacterial viability during storage (Jin et al. 1985; Simpson et al. 2005). The matrix constituents were found to play an important role in the survival of *Lactobacillus paracasei* during storage. The protective carbohydrates present in the probiotic carrier,

in amorphous glassy state, play an important role by limiting the membrane lipid oxidation, protein unfolding and chemical reactions, by providing an effective environmental barrier and thereby minimizing the transitional molecular motion (Sun and Leopold, 1997; Crowe et al. 1998).

MPI consists of mainly proteins (90%) and is deficient in both lactose and fat (3%). The loss of viability was observed in MPI powders with 0.3 $a_{w,}$ from 9.75±0.15 to 8.24±0.12 log CFU/g and 0.4 $a_{w,}$ from 9.93±0.12 to 5.72±0.14 log CFU/g upon storage at 25˚C. The viability loss in these powders may be attributed to the presence of no protective carbohydrate (lactose) in the matrix, which provides protection during drying or desiccation/drying stress. Therefore, the increased bacterial death in MPI matrix during storage, compared to other matrices may be due to the greater cell injury during the drying process (osmotic stress) in the absence of protective carbohydrate. This may result in increased number of bacteria dying off during the storage. Furthermore, there would also be the effect of higher water activity that is known to increase translational molecular motion leading to oxidative damage and bacterial viability loss in the powders on storage.

SMP possess high amount of lactose 54.5%, 32.9% protein and 0.9% fat, while WMP contains 39.1% lactose, 25% protein and 26.8% fat. In the case of bacteria embedded in SMP and WMP matrices, high amount of lactose would provide protection to the bacterial cells during the drying process by forming an amorphous glass. The amorphous glassy state of carbohydrate imparts very high viscosity at glass transition temperatures and thereby restricts the molecular mobility in the matrix. Lactose has been found to be effective in maintaining the structural and functional integrity of model membranes (microsomes) at low a_w (glassy state). The combination of a_w 0.43

and T=4.8± 1.5ºC, 0.395 a_w and T=25ºC, 0.3 a_w and T=38ºC is considered as border point, as below this temperature/water activity combination lactose remains in the glassy state. The crystallization may therefore not be present in the bacterial samples stored at 0.3 and 0.4 a_w, in line with previous reports (Thomsen et al 2005). Moreover, the presence of proteins in the matrix would also help in retaining the glassy form of lactose in the SMP and WMP matrices. The presence of fat in the WMP matrix may offer hydrophobic barrier thereby helping in maintaining the protective glassy state. It has been previously observed that encapsulation material containing fat improves the viability of bacteria at high water activities (Crittenden et al. 2006). The WMP matrix provided better protection during storage at elevated storage temperature compared to SMP and MPI matrix where no viable bacterial count could be observed after 4 week storage. This observed protective effect may be due to the presence of fat in the WMP matrix.

3.5 Conclusions and future research

Whole milk powder matrix may have provided better protection, due to the presence of appropriate proportions of fat, protein and lactose. Therefore, to gain further mechanistic insights, bacteria would be further embedded in WMP matrix, and the effects of drying method and storage of the powders under varying water activity conditions will be studied. The proposed study in Chapter 4 may shed some further light on the protective mechanisms of matrix component and interrelationship between bacterial death and storage water-activity. Irrespective of the embedding matices and powder water activity, bacterial viability was maintained during storage at 4ºC, while high loss occurred upon storage at 37ºC. Hence, the powders under these storage temperatures conditions would not be studied further. The present study hinted the

importance of fat in the drying matrix which would also be explored in detail in Chapter 4. The glassy state of lactose and its relationship with bacterial viability will also be explored in Chapter 4.

Chapter 4 Effect of drying techniques (freeze drying, spray drying and fluidized bed drying) on the viability of probiotic *Lactobacillus paracasei*

4.1 Introduction

In order to stabilize probiotic bacteria for long term storage, a right combination of stabilization technology, drying method, matrix constituents and matrix architecture is necessary. Fluidized bed drying of *L. paracasei* in a whole milk powder (WMP) matrix was found to provide better protection in Chapter 3. In this chapter, additional drying techniques, i.e. freeze drying and spray drying were evaluated alongside fluidized bed drying, using WMP as the embedding matrix. In the past, stabilization technologies, such as freeze drying and spray drying have been used for the production of concentrated probiotic powders. However, limited success has been observed in maintaining bacterial viability under ambient temperature conditions, simulating non-refrigerated food matrix environment (Miao et al. 2008). In the case of freeze drying process, there exists an increased loss in bacterial viability during the freezing step due to the ice crystal formation and the rupturing of the cell membrane (Fonseca et al. 2004). Similarly, high temperature of spray drying process results in thermal inactivation of cells (Teixeira et al. 1995; Fonseca et al. 2000; Ananta et al. 2005). Probiotics are sensitive to temperature, such that the death rate typically increases with drying temperature (Gardiner et al. 2002), which is typically above 51ºC (Simpson et al. 2005). As a result, several process optimization efforts have been carried out to reduce the bacterial death by minimizing the drying temperature (Gibbs et al. 1999; Gardiner et al. 2002; Kailasapathy, 2002; Ananta and Knorr, 2003).

The fluidized bed drying process on the other hand utilizes mild temperature, minimizing bacterial inactivation by using rapid exchange of heat and mass between the particles (Bayrock and Ingledew, 1997). This process has been widely used for the production of baker's yeast and wine yeast, but to date it has found limited application in the production of concentrated viable bacteria (Caron, 1995).

The matrix within which the probiotic bacteria are contained is important for the stability of the bacteria. Carbohydrates, protein and fat may all play a protective role for the bacteria. Carbohydrates, such as lactose, can be used in the drying matrix and act to substitute for the hydrogen-bonded water in the head group of the phospholipid bilayers present in the bacterial cell membrane (Leslie et al. 1995; Morgan et al. 2005; Santivarangkna et al. 2008). In addition, if the carbohydrates form a glassy (amorphous) state during drying, they can impart very high viscosity, which can act as a protective encapsulation for the bacteria, limiting water and oxygen exchange (Morgan et al. 2006). Proteins can delay the crystallization of carbohydrates to maintain the protective glassy state (Haque and Roos, 2004; Jouppila and Roos, 1994; Shrestha et al. 2007). Similarly, fat may also delay the crystallization of lactose (Kim et al. 2003; Knudsen et al. 2002). Therefore, the composition and the structure of the matrix play important roles in increasing the storage life of dried bacteria.

The viability of bacteria during storage depends on moisture content, which in turn is influenced by the water activity conditions during storage (Gunning et al. 1995; Hsiao et al. 2004; Miao et al. 2008). At a water activity below 0.11 a_w *Lactobacillus* can be kept viable for long periods (Gunning et al. 1995; Miao et al. 2008). A glassy (non-crystalline) state has been shown to enhance the storage life (Ananta et al. 2005; Crowe et al.1998; Miao et al. 2008; Sun and Leopold, 1997) but, at higher humidity, water be-

gins to act as a plasticizer of the protective carbohydrate glass and increases the molecular mobility (Thomsen et al. 2005). This increase in molecular mobility results in crystallization of the amorphous carbohydrate and instantaneous loss of bacterial viability during storage (Miao et al. 2008).

Stabilization technologies, such as freeze drying and spray drying, have been used previously for the production of concentrated probiotic powders. However, there has been limited success in maintaining bacterial viability under ambient temperature conditions. The current study was planned to investigate the effects of drying technology and the storage water activity on the viability of *Lactobacillus paracasei* 431embedded in a whole milk matrix. Moreover, impact of the matrix structure on the viability of the bacteria was also explored.

4.2 Materials and methods

Whole milk powder (WMP) was obtained from Fonterra Co-operative Group Ltd, New Zealand.

4.2.1 Bacterial growth and cell harvesting conditions

Lactobacillus paracasei subsp. *paracasei* CRL 431 (ATCC 55544) was grown batch-wise in a 5 L durham bottle in 55 gL^{-1} MRS medium (Merck KGaA, Darmstadt, Germany) at 37 ºC. The cells in the stationary phase 18 h after inoculation were harvested by centrifugation (10 min at 15000g, in a Sigma 6-16S centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The harvested cells were washed in (5 gL^{-1} buffered peptone water, (Merck), resulting in a cell pellet containing ~ 3 x 10^{11} CFU/g of dry pellet. WMP was reconstituted in purified water to 35% (w/w) and then the freshly harvested bacterial pellets were dispersed into reconstituted milk for 30 min prior to

the spray drying- or freeze drying process; this resulted in 1.1% *L. paracasei* in the milk powder on a dry weight basis. For fluidized bed drying, the harvested cells were mixed manually with whole milk powder for 10 min to form a uniform mass before drying.

4.2.2 Drying processes

Three types of drying technologies were used in the present study: spray drying, freeze drying and fluidized bed drying.

4.2.2.1 Spray drying

Spray drying process comprises of the atomization of a liquid feed into a spray of droplets and upon contact with hot air in the drying chamber; these droplets forms dried powders. The spray drying process was carried out on a pilot-scale spray drier (model MOBILE MINOR, GEA Niro, Søborg, Denmark (Figure 4.1)) using a co-current spray nozzle with an inlet air temperature of 100 ºC and an outlet temperature of 45−48 ºC, manually adjusted by controlling the feed flow rate (Chavez and Ledeboer, 2007; Hsiao et al. 2004). The probiotic bacteria are sensitive to temperature and therefore the spray drier setting was optimized to co-current airflow setting. In co-current setting, the drying air and particles move through the drying chamber in the same direction. This reduces the heat damage as compared to the counter current airflow settings. The inlet air temperature was lowered to 100ºC, which resulted in lower outlet temperature between 45-48ºC, thereby minimizing the heat damage on the cells (Hsiao et al. 2004; Chavez and Ledeboer, 2007).

4.2.2.2 Freeze drying

Freeze drying process involves a process known as sublimation, whereby water is removed from solid to gaseous state without the formation of liquid state. In the freeze

drying process the products are firstly deep frozen and then dried. For freeze drying, the whole milk−bacteria suspension was spread evenly into a thin film and frozen at −80 ºC in plastic bags. Freeze drying was carried out subsequently in a pilot-scale freeze drier (model FD18LT, Cuddon, Blenheim, New Zealand (Figure 4.2)) at 0.2 mbar vacuum for 96 h, with a start temperature of ~30 ºC and an end temperature of 25 ºC.

Figure 4-1 Image of spray drier, model MOBILE MINOR, GEA

Figure 4-2 Image of freeze drier, model FD18LT, Cuddon

4.2.2.3 Fluidized bed drying

In the fluidized bed drying process air travels through the bed of particles with sufficient velocity to provide fluid like behavior, and the freely suspended particles in the air stream are dried by the rapid exchange of heat and mass transfer. The whole milk−bacteria mixture was dried in a laboratory fluidized bed drier (model Uni-Glatt, Glatt GmbH, Binzen, Germany) with dehumidified compressed air at 40 ºC (refer to Chapter 3).

4.2.3 Particle size distribution, water activity and moisture content

4.2.3.1 Particle size distribution

The particle size distribution of the dried-probiotic-containing powder was measured using a Malvern Mastersizer 2000 instrument (Malvern Instruments Ltd, Malvern, UK). The Mastersizer 2000 uses the principle of laser diffraction to measure the size of particles. It measures the intensity of light scattered as the laser beam passes through a dispersed particulate sample. This data is then analyzed to calculate the size of the particles derived from the scattering pattern. The Scirocco 2000 used in the study is an automated dry powder dispersion unit for the Mastersizer 2000 particle size analyzer. In case of dry powder dispersion, the dispersant is usually a flowing gas stream; in this study it was clean dry air.

4.2.3.2 Water activity and moisture content analysis

Water activity (a_w) was measured using a Decagon AquaLab 4TE water activity instrument (Decagon Devices Inc., WA, USA) at 25 °C. The moisture content from 1 g of sample was measured after drying for 3 h at 102 ± 2 ºC in a ventilated drying oven Contherm Series 5 (Contherm Scientific Limited, Upper Hutt, Wellington, New Zealand) and cooling for 60 min in a desiccator containing silica desiccant (Isengard, 2008). The

term moisture content and water content have been often used interchangeably to represent the quantity of water in a given product. The moisture content provides information about texture of amorphous carbohydrates (lactose in case of dairy matrix), since increasing levels of moisture provides water mobility and lowers the glass transition temperature. Water activity represents the energy status of the water in the system. It is defined as the ratio of the vapor pressure of water in a sample to the vapour pressure of pure water at the sample temperature. Water activity and moisture content may provide a better understanding of the water in the system.

4.2.4 Storage conditions

All samples of dried powder were immediately stored in desiccators under vacuum at 25 ºC and under the following conditions: 0.11 a_w (saturated aqueous LiCl); 0.33 a_w (saturated aqueous $MgCl_2$); 0.52 a_w (saturated aqueous $Mg(NO_3)_2$). These a_w values are similar to those encountered in tropical and moist climates, under closed and open can condition (Weinbreck et al. 2010). The analytical grade salts were obtained from Thermo Fisher Scientific New Zealand Ltd, Auckland, New Zealand.

4.2.5 Enumeration of viable bacteria

After a 10-day equilibration period, powder samples were prepared each week, over a total period of 105 days, for the enumeration of viability by homogenization in sterile buffered peptone water (5 gL-1, Merck) for 5 min using a Stomacher 400 Lab Blender (Seward Medical, London, UK). From this homogenate, 10-fold serial dilutions were made in the same sterile buffered peptone water and were used for microbiological analyses, and the diluted samples were pour plated on MRS agar plates (Merck). Viable

cell counts were determined after 48 h of incubation under aerobic conditions at 37 ºC. The colonies counted were recorded as colony-forming units per gram of product.

4.2.6 Characterization of powders

4.2.6.1 Helium pycnometry

The pore volumes of the powders were measured with a AccuPyc 1330 pycnometer (Micromeritics, Norcross, GA, USA). At first the solid volume of the sample was measured and thereafter the sample was crushed to expose isolated pores. The resulting measure of specimen volume is combined with an accurately determined specimen mass to obtain the skeletal density. The system was calibrated before each measurement using a reference sphere, and each measurement was repeated 10 times or until the standard deviation of the results was below 0.05%.

4.2.6.2 Scanning electron microscopy

In a scanning electron microscopy, a beam is focused onto the sample surface kept in a vacuum by electro-magnetic lenses; thereafter the beam is scanned over the surface of the sample. The scattered electrons from the sample are fed into the detector then to a cathode ray tube through an amplifier, where the images are formed providing valuable information related to surface topology. For this study, the powders were mounted on double-sided tape and sputter coated with gold, and digital images were recorded with a Quanta 200 scanning electron microscope (FEI, Hillsboro, OR, USA). Digital images were captured and saved at the required magnification.

4.2.6.3 Confocal laser scanning microscopy

The structure of the powders was studied using a TCS 4D confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). The microscope used for this study is fitted with means to acquire 12-bit images using four distinct detectors for fluo-

rescent signals from fluorophores excited by four lasers (Argon, DPSS, HeNe, UV) and a transmission detector for bright-field images (DIC). Briefly, the powder sample was prepared for microscopy by mixing powder sample with Syto9/Syto 24 and Propidium iodide (PI) stain solution, followed by overnight incubation at 25°C before analysis. Syto9 solution was prepared by adding 20 μL of 500 mmol syto9 solution (Invitrogen) to 960 μL of glycerol and 20 μL of water), while the PI solution was made by adding 20 μL of 1mg/ml PI (Sigma) stock solution to 960 μL of glycerol and 20 μL of water. Glycerol was found to leave the powder structure intact, while the water aided in the diffusion of the dye in the samples.

4.2.6.4 X-ray diffraction

The interaction of the X-ray beam with the crystal in the sample causes diffraction. The angle of diffraction provides information regarding the crystal nature of the substance. X-ray diffraction is a useful technique to determine the presence of crystals in a sample. Crystals are solids which are exact repeat of symmetric motif. Molecules in crystals are arranged in symmetrically as compared to those in amorphous solids, where the arrangements are random. X-ray powder diffraction is an optimal tool to differentiate between amorphous lactose and crystalline lactose in the powders. The X-ray diffraction patterns of powdered samples were recorded on a PW1840 diffractometer (Philips, Amesterdam, The Netherlands) using Co Kα radiation.

4.2.6.5 Thermogravimetric analysis/Differencial scanning calorimetry (TGA/DSC)

Thermal gravimetric analysis was conducted using a Q600 (TA instruments, USA), which provides simultaneous measurement of the weight change (TGA) and the true differential heat flow (DSC) on the same sample. Each bacterial powder sample stored

at different water activity was accurately weighed into 50 μL alumina crucible using the TGA/SDTA microbalance. The powder sample was heated from 25 to 300°C at a heating rate of 5°C.min^{-1}; under nitrogen flush (50 mL.min^{-1}). The instrument measured the change in mass and recorded the temperature profile and was calibrated using indium. Data were analyzed using the TA Advantage software for windows (TA instruments, USA).

In thermogravimetric analysis, the amount and rate of change in the mass of a sample is measured, while the sample is heated at a constant rate (or at a constant temperature) in a controlled atmosphere (under air or nitrogen).TGA is effective in providing information of mass changes such as evaporation, dehydration etc. which was used to validate the moisture content analysis of the samples.

Differential scanning calorimetry (DSC) typically measures the amount of heat absorbed or released by a sample as it is heated or cooled or kept at constant temperature (isothermal), ie. when the sample undergoes a physical transformation, such as phase transitions, more (or less) heat will need to flow to it than the reference to maintain both at the same temperature. The sample and reference material are simultaneously heated or cooled at a constant rate. The difference in temperature between them is proportional to the difference in heat flow (from the heating source i.e. furnace) between the two materials. When a sample undergoes exothermic processes (such as crystallization) less heat is required to raise the sample temperature. By monitoring the difference in heat flow between the sample and reference, differential scanning calorimeter measures accurately the amount of heat absorbed or released during such transitions.

Statistical analysis was carried out as described in 4.2.6.9.

4.2.6.6 Fourier transform infrared (FT-IR) spectroscopy

FT-IR encompasses the absorption of electromagnetic radiation in the infrared region of the spectrum, leading to changes in the vibrational energy of molecule. All molecules within a compound would have vibrations in the form of stretching, bending, etc., the absorbed energy thereby leads to a change in energy levels. Fourier transform infrared spectroscopy is an easy way to determine the functional group in a molecule and study changes in them. It simultaneously collects spectral data in a wide spectral range. FT-IR is able to resolve fat, protein, lactose and water in WMP embedding matrix. Attenuated total reflectance (ATR) spectra were acquired using a germanium- ATR crystal mounted in a nitrogen purged Nicolet 5700 FT-IR (Thermo Electron Scientific Instruments Corp., Madison, WI USA) with H_2O and CO_2 background subtractions. Bacteria-containing powdered samples were kept in good optical contact on the germanium surface. The system was operated under OMNIC version 7.1, and Spectra of 128 scans were taken with a spectral resolution of 4 cm^{-1}. Three spectra of individual samples were recorded, a total of 27 ATR spectra of bacteria-containing powdered samples were recorded one month after manufacture and storage under conditions of controlled water activity.

Statistical analysis was carried out as described in 4.2.6.9.

4.2.6.7 Raman spectroscopy

Raman spectroscopy provides complementary information to that provided by FT-IR. Raman spectroscopy is based on the inelastic scattering of laser light which interacts with the electron cloud and the bond of a molecule. Here, a laser beam (near-IR region) is directed to the sample and the scattered radiation is collected. Most of the scattered radiation has the same wavenumber as that of the incident laser beam, while a fraction

has a different wavenumber. The photon excites the molecule from a ground state to a virtual energy state, and while relaxing the molecule emits a photon and returns to a different rotational or vibrational state. This is the Raman signal and characteristic of particular functional group and provides information on the structure of the molecule. For Raman spectroscopic analysis, the powder was packed tightly into divots and smoothed on top to give a flat measurement surface. Raman spectroscopy was carried out on the Bruker Equinox 55 interferometer equipped with the Bruker FRA 106/S FT-Raman accessory (Bruker corporation, Billerica, Massachusetts (USA)), with the source Nd:YAG laser set at 1064 nm. The focused aperture setting giving 300 µm diameter spot size was used, with the 120 mW power setting, 128 scans per spectrum and spectral resolution of 2 cm^{-1}. Each sample was measured in triplicate

Statistical analysis was carried out as described in 4.2.6.9.

4.2.6.8 Nuclear Magnetic Resonance (NMR)

Spatial proximity and chemical bonding between atoms leads to interactions between nuclei. These interactions are dependent on the orientation of the molecule. In solid medium such as crystals and powders, anisotropic interactions influence the behavior of a system of nuclear spins. The, solid-state NMR spectra are very broad, because the full effects of anisotropic or orientation-dependent interactions are observed in the spectrum. Magic angle spinning introduces artifical motion by placing the sample rotor at a magic angle (54.74), when combined with cross polarization, polarization from the abundant nuclei like ^{1}H can be transferred to dilute rare nuclei like ^{13}C in order to enhance signal to noise. The ^{13}C solid state NMR spectrum is very useful technique to study molecular mobility. It reflects the fact that every molecule has a magnetic environment which repeats regularly so as to produce a rather narrow dispersion of chemical

shifts. On the contrary, in the presence of molecular disorder, the dispersion is larger due to the large number of environments, and moreover is variable with time.

Solid state ^{13}C MAS NMR with cross polarization (CP) was used to characterize the changes that occur in the matrix during storage. The NMR analysis was conducted in a Brucker (Rheinstetten, Germany) DRX 200MHz horizontal bore magnet. Finely ground samples were packed into a 7 mm diameter cylindrical zirconia rotor with Kel-F end caps and spun at speeds of 5.0 ± 0.2 kHz in a dual resonance magnetic angle spinning (MAS) probe from Doty Scientific. During acquisition the sample temperature was maintained at 20 °C. Solid-state ^{13}C MAS NMR spectra were obtained at a ^{13}C frequency of 50.3 MHz on a Bruker (Rheinstetten, Germany) DRX 200 MHz spectrometer. Free induction decays FIDs were acquired with a sweep width of 30 KHz; 960 data points were collected over an acquisition time of 30 ms. The CP-MAS spectra were acquired with a ^{1}H 90° pulse for 5.5 µs, a cross-polarization contact time of 1000 µs, an acquisition time of 30 ms, relaxation time of 2 s and 4 k scans. All spectra were zero filled to 4 k data points and processed with a 0.005 s Gaussian broadening. Chemical shifts were externally referenced with glycine.

4.2.6.9 Statistics – principal component analysis (PCA)

PCA is a useful statistical technique and is a common technique for finding patterns in data of high dimension. The TGA/ DSC, FT-IR, Raman spectroscopic data was simplified using PCA which aided in better interpretation of results. For PCA processing, all the acquired spectrums were converted to “csv” format and pre-processed for PCA by standard normal variate (SNV) normalisation. Normalization and PCA were performed using the R program (Wehrens and Mevik 2007). Score plots of the first few PCs were constructed with color-coding of the replicated samples to exhibit the achieved within-

and between-sample separation. To obtain details on the components of the spectra that are separating samples, the loadings plots for each relevant PC were further analyzed to identify the peaks contributing to the separation.

Since patterns in data can be hard to find in data of high dimension, where the graphical representation is not available, PCA is a powerful tool for analyzing data. Once the patterns in the data are found the data can be compressed by reducing the dimensions without much loss of information. PCA analysis carried out in this study is descriptive, of exploratory nature, and the description certainly showed separation between the groups. Typical R- program used in the study to carry out PCA of the FT- IR spectrum is shown below.

```
# READ IN DATA:
Spectra<-read.csv("PCA.CSV")
head(Spectra)
tail(Spectra)
n<-dim(Spectra)[2]
r<-3   # Number of replicates - change as necessary

# PLOT WITH/WITHOUT NORMALIZING:
par(mfrow=c(2,1))
matplot(Spectra[,1],Spectra[,2:n],type="l",xlab="wave no.",ylab="Absorbance",main="Raw")
X<-scale(Spectra[,2:n],center=TRUE,scale=TRUE)
matplot(Spectra[,1],X,type="l",xlab="wave no.",ylab="Absorbance",main="Normalized")

# REMOVE INERT WAVELENGTHS:
SpectraR<-Spectra[(Spectra[,1]<3600),]
SpectraR<-SpectraR[(SpectraR[,1]<1900)|(SpectraR[,1]>2750),]
SpectraR<-SpectraR[(SpectraR[,1]>=750),]
```

```
head(SpectraR)
tail(SpectraR)
```

 # RESCALE AFTER REMOVAL:

```
X<-scale(SpectraR[,2:n],center=TRUE,scale=TRUE)
matplot(SpectraR[,1],X,type="l",xlab="wave no.",ylab="Absorbance")
```

 # PLOTTING AFTER REMOVAL:

```
Waxis<-SpectraR[,1]-850*(SpectraR[,1]>1900)
par(mfrow=c(2,1))
matplot(Waxis,X,type="l",xaxt="n",xlab="Wave no.",ylab="Absorbance",main="Scaled")
axis(1, at=c(1000,1500,1900,2150,2650),labels=c(1000,1500,"*",3000,3500))
abline(v=1900)
matplot(Waxis,SpectraR[,2:n],xaxt="n",type="l",xlab="wave
no.",ylab="Absorbance",main="Unscaled")
axis(1, at=c(1000,1500,1900,2150,2650),labels=c(1000,1500,"*",3000,3500))
abline(v=1900)
# PCA USING PRCOMP():
SpectraR.pca<-prcomp(t(X))
summary(SpectraR.pca)
screeplot(SpectraR.pca)
LoadMat<-SpectraR.pca$rotation
ScoreMat<-predict(SpectraR.pca)
#locator()
> row.names(t(X))
 [1] "FBD.0.1"   "FBD.0.1.1" "FBD.0.1.2" "FBD.0.3"   "FBD.0.3.1"
"FBD.0.3.2" "FBD.0.5"
 [8] "FBD.0.5.1" "FBD.0.5.2" "FZD.0.1"   "FZD.0.1.1" "FZD.0.1.2"
"FZD.0.3"   "FZD.0.3.1"
[15] "FZD.0.3.2" "FZD.0.5"   "FZD.0.5.1" "FZD.0.5.2" "SPD.0.1"
```

```
"SPD.0.1.1" "SPD.0.1.2"
[22] "SPD.0.3"   "SPD.0.3.1" "SPD.0.3.2" "SPD.0.5"   "SPD.0.5.1" "SPD.0.5.2"
Symbol<-c(0,0,0,1,1,1,2,2,2,15,15,15,16,16,16,17,17,17,5,5,5,6,6,6,8,8,8)
Colour<-c("black" ,"black" ,"black" ,"black" ,"black" ,"black" ,"black" ,"black" ,"black",
"black" ,"black" ,"black" ,"black" ,"black" ,"black" ,"black" ,"black" ,"black","black" ,
"black" ,"black" ,"black" ,"black" ,"black" ,"black")
par(mfrow=c(2,1))
plot(Waxis,LoadMat[,1],type="l",xaxt="n",xlab="Wave
no.",ylab="Loadings",main="PC1",ylim=c(-0.15,0.2),col="blue",lwd=3)
abline(h=0)
axis(1, at=c(1000,1500,1900,2150,2650),labels=c(1000,1500,"*",3000,3500))
abline(v=1900,lty=2)
plot(Waxis,LoadMat[,2],type="l",xaxt="n",xlab="Wave
no.",ylab="Loadings",main="PC2",ylim=c(-0.15,0.2),col="blue",lwd=3)
axis(1, at=c(1000,1500,1900,2150,2650),labels=c(1000,1500,"*",3000,3500))
abline(v=1900,lty=2)
abline(h=0)
locatorplot(ScoreMat[,1],ScoreMat[,2],type="n",xlab="PC1",ylab="PC2")
for (i in 1:(n-1))
{ text(ScoreMat[i,1],ScoreMat[i,2],row.names(ScoreMat)[i],cex=1.5,col=ceiling(i/r)) }
plot(ScoreMat[,1],ScoreMat[,2],xlab="PC1 (46%)",ylab="PC2
(34%)",pch=Symbol,col=as.character(Colour)
,bty="l",cex=2)
abline(h=0,lty=2)
abline(v=0,lty=2)
legend("topleft",inset=0.02,
legend=c("FBD12","FBD32","FBD52","FZD12","FZD32","FZD52","SPD12","SPD32","SPD5
2"),
pch=rep(c(0,1,2,15,16,17,5,6,8),c(1,1,1,1,1,1,1,1,1)),col=rep(c("black"),9))
abline(h=0,lty=2)
```

```
abline(v=0,lty=2)
```

Three batches of powders were made for each drying process; two batches were fully analysed for chemical and structural analysis. The results shown in the present study are that of batch 1. The results obtained from other batches showed similar trends to that obtained from batch 1.

4.3 Results and Discussion

4.3.1 Storage stability

The goal of this work was to understand the impact of drying methods on the survival of *L. paracasei* in a whole milk powder matrix during storage at 25 °C. Three drying methods, i.e. freeze drying, spray drying and fluidized bed drying, were compared. Immediately after drying, the bacterial viability was measured and the plate counts of the freeze dried powder, spray dried powder and fluidized bed dried powder were 9.94 ±0.03 log CFU g^{-1}, 9.76± 0.05 log CFU g^{-1} and 9.27± 0.03 log CFU g^{-1}, respectively (Figure 4.3 a-c). For the spray drying and freeze drying process, the bacterial pellet was dispersed in the reconstituted whole milk powder before drying. Therefore, the differences in the initial count between spray- and freeze dried powder probably reflect the small loss of viability due to temperature rise during spray drying, although relatively low inlet and outlet temperatures were used. However, in case of fluidized bed dried powder, the bacterial pellet was mixed manually with dry milk powder in a separate batch process. Therefore, the slightly lower initial count was probably due to some variability in handling and transferring bacterial pellets into milk powder and/or losses during fluidized bed drying itself.

The viability counts decreased during the equilibration process and storage thereafter at 25 °C. After 10 d of equilibration period (baseline), freeze dried powder stored at a_w values of 0.11, 0.33 and 0.52 had viability counts of 9.63±0.09 log CFU g^{-1}, 8.70±0.10 log CFU g^{-1} and 6.87±0.21 log CFU g^{-1} respectively. Under the same conditions, spray dried powder stored at a_w values of 0.11, 0.33 and 0.52 had viability counts of 9.45±0.13 log CFU g^{-1}, 8.69±0.05 log CFU g^{-1} and 7.47±0.49 log CFU g^{-1} respectively, whereas fluidized bed dried powder stored at a_w values of 0.11, 0.33 and 0.52 had viability counts of 9.17±0.17 log CFU g^{-1}, 9.16±0.08 log CFU g^{-1} and 8.13±0.11 log CFU g^{-1} respectively. The powders that had been stored at 0.11 a_w showed a slow loss of viability over the 105 d regardless of the drying method. By contrast, when stored at 0.52 a_w, all powders showed a complete loss of viability within 22 d. At the intermediate 0.33 a_w, there were marked differences in bacterial viability between the three drying methods. The fluidized bed dried powder showed the slowest decline in viable bacterial count during the 105-day period, 9.27± 0.03 to 7.91± 0.07 log CFU g^{-1}, the freeze dried powder showed the fastest decline, 9.94±0.03 to 4.64±0.05 log CFU g^{-1} and the spray dried powder was intermediate, 9.76±0.05 to 7.64±0.15 log CFU g^{-1} (Figure 4.3 a-c). As expected, for any individual drying method, the humidity conditions during storage of the dried powders were important, with higher humidity leading to a loss of bacterial viability. What we need to understand is how the differences in storage stability between the powders produced by different drying methods arise.

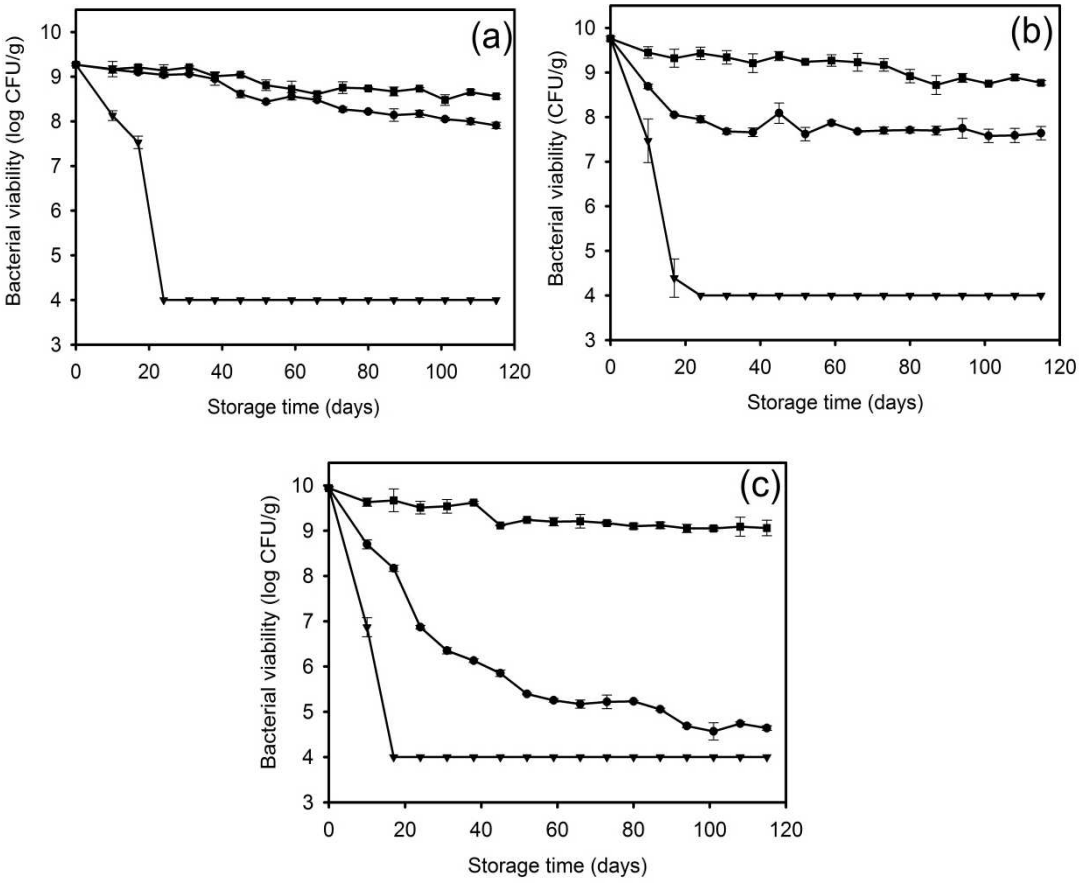

Figure 4-3 Effect of drying techniques on the viability of *L. paracasei* powders during storage at 25 °C under controlled water activity conditions: (a) fluidized bed drying; (b) spray drying; (c) freeze drying; squares, 0.11 a_w, circles, 0.33 a_w; triangles, 0.52 a_w. Error bars represent stand deviation of means (n≥3)

4.3.2 Moisture content

The moisture contents of the powders obtained by each of the three methods were different (Table 4-1). The freeze dried material had the lowest moisture content, the spray dried material had the highest moisture content while the fluidized bed dried material had an intermediate moisture content. On equilibrating at 0.11 a_w or 0.33 a_w, the moisture content increased or decreased depending on whether the equilibration humidity was less or greater than the initial moisture content. However, the situation was differ-

ent when equilibration took place at 0.52 a_w; in this case, the moisture content was less than that for the powders equilibrated at 0.33 a_w, suggesting higher proportions of water in the sample became more strongly bound, e.g. as water of crystallization.

Table 4-1 Moisture content of powders prepared using different drying techniques and containing *L. paracasei* 431 after equilibration at different water activity.

Drying technology	Moisture content (%)	a_w before equilibration	Moisture content after equilibration at 0.11 a_w (%)	Moisture content after equilibration at 0.33 a_w (%)	Moisture content after equilibration at 0.52 a_w (%)
Fluidized bed drying	3.94 ± 0.14[a]	0.280	1.67 ±0.19 [a]	4.81 ±0.02 [a]	4.17 ±0.14 [a]
Spray drying	7.17 ± 0.09[a]	0.470	2.56 ± 0.04 [a]	5.11 ±0.03 [a]	4.54 ±0.05 [a]
Freeze drying	1.67 ± 0.19[a]	0.098	2.49 ± 0.13 [a]	5.43 ±0.08 [a]	4.57 ±0.09 [a]

[a] Error values represent stand deviation of means (n≥3)

These results suggest that the initial moisture contents of the dried powders do not seem to be an important factor in the long term storage viability of the *L. paracasei*. Previous studies have shown the importance of the moisture content in the viability of bacteria in powders during storage, with an increase in moisture content leading to a rapid loss of bacterial viability (Gunning et al. 1995; Hsiao et al. 2004; Miao et al. 2008). It was observed that the moisture contents of the powders stored at 0.11 a_w were in the range 1.7−2.6% and, at this low level, the bacterial viability was retained for long periods. At 0.33 a_w, the moisture contents of the stored powders increased to 4.8–5.4%, which ad-

versely affected the bacterial viability; small differences in moisture led to large differences in the bacterial viability loss during the storage period (Table 4-1). High bacterial viability count was observed during storage at 0.11 a_w among the powders and freeze dried powder had higher amount of live bacteria. However, the problem arises when freeze dried probiotics are applied into products such as infant formula, breakfast cereals, muesli etc. These food products have inherent a_w values ranging from 0.2-0.4 and the present results indicate that the fluidized bed dried powder may be more effective ingredient solution for such applications, as it takes up lower moisture (fluidized bed drying, 4.81% vs freeze drying 5.43% at 0.33 a_w). At higher a_w during storage (0.52 a_w), the moisture content continued to increase, with complete loss of bacterial viability lost among the powders within 21 days of storage.

4.3.3 Powder morphology and porosity

The three drying methods resulted in different powder morphologies. The fluidized bed dried powder had a structure consisting of agglomerated spheres, the spray dried powder showed largely individual spherical particles while the freeze dried powder had a porous-sheet-like structure (Figure 4-4). The particle size of the fluidized bed dried powder (estimated using light scattering) was mostly in the range 100–700 µm whereas that of the spray dried powder was 20–100 µm. For the freeze dried material, the particle size was dependent on the grinding post treatment; in this study, the particle size was mostly in the range 100–1000 µm. The fluidized-bed- and spray dried materials had similar bulk porosities of 50.3 and 51.5% respectively. The connected porosity for these powders was approximately 38%. However, the freeze dried material had a higher bulk porosity of 77.5%, most (71.2%) of which comprised connected pores (Table 4-2; Figure 4-5).

These differences in morphology, including the porosity and the structure, of the powders could explain the variations in moisture content after storage in controlled humidity environments between the powders produced by different drying methods. The moisture content after equilibration at controlled humidity was highest for the freeze dried material and lowest for the fluidized bed dried material (Table 4-1). It is likely that water was primarily absorbed initially to powder surfaces. The surface area for water absorption depends on both the particle size and the porosity. The fluidized bed dried powder had the lowest surface area, which was probably why it had the lowest moisture content after storage in a controlled humidity environment. By contrast, the freeze dried material had the highest connected porosity, providing a large internal surface area for water absorption, which may have been the reason for its high moisture content after storage in a controlled humidity environment.

Table 4-2 Porosities, obtained by helium pycnometry, of various powders containing *L. paracasei* 431.

Sample	Connected porosity (%)	Isolated porosity (%)	Bulk porosity (%)
Fluidized bed dried	38.5	11.8	50.3
Spray dried	38.0	13.5	51.5
Freeze dried	71.2	6.3	77.5

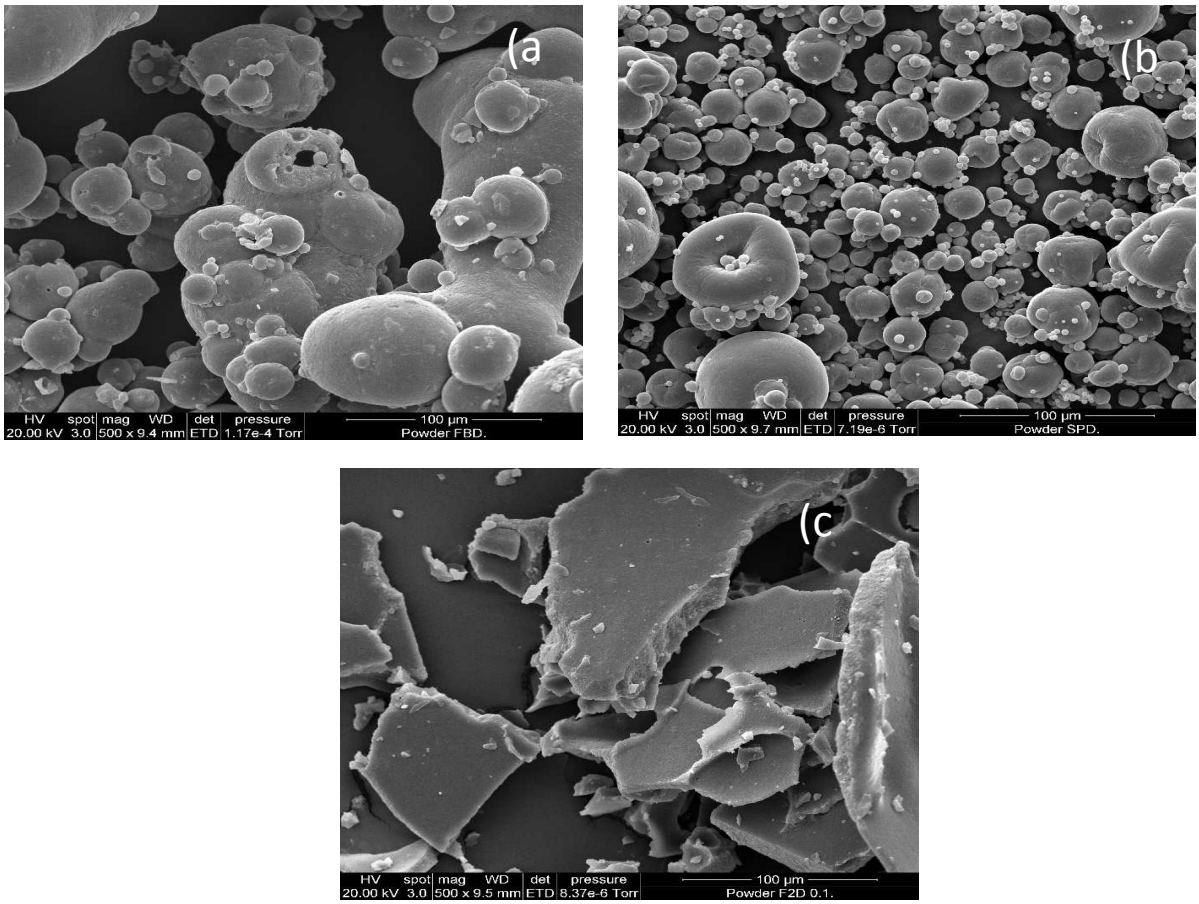

Figure 4-4 Scanning electron microscopy images of powders containing *L. paracasei* 431 obtained by (a) fluidized bed drying, (b) spray drying and (c) freeze drying.

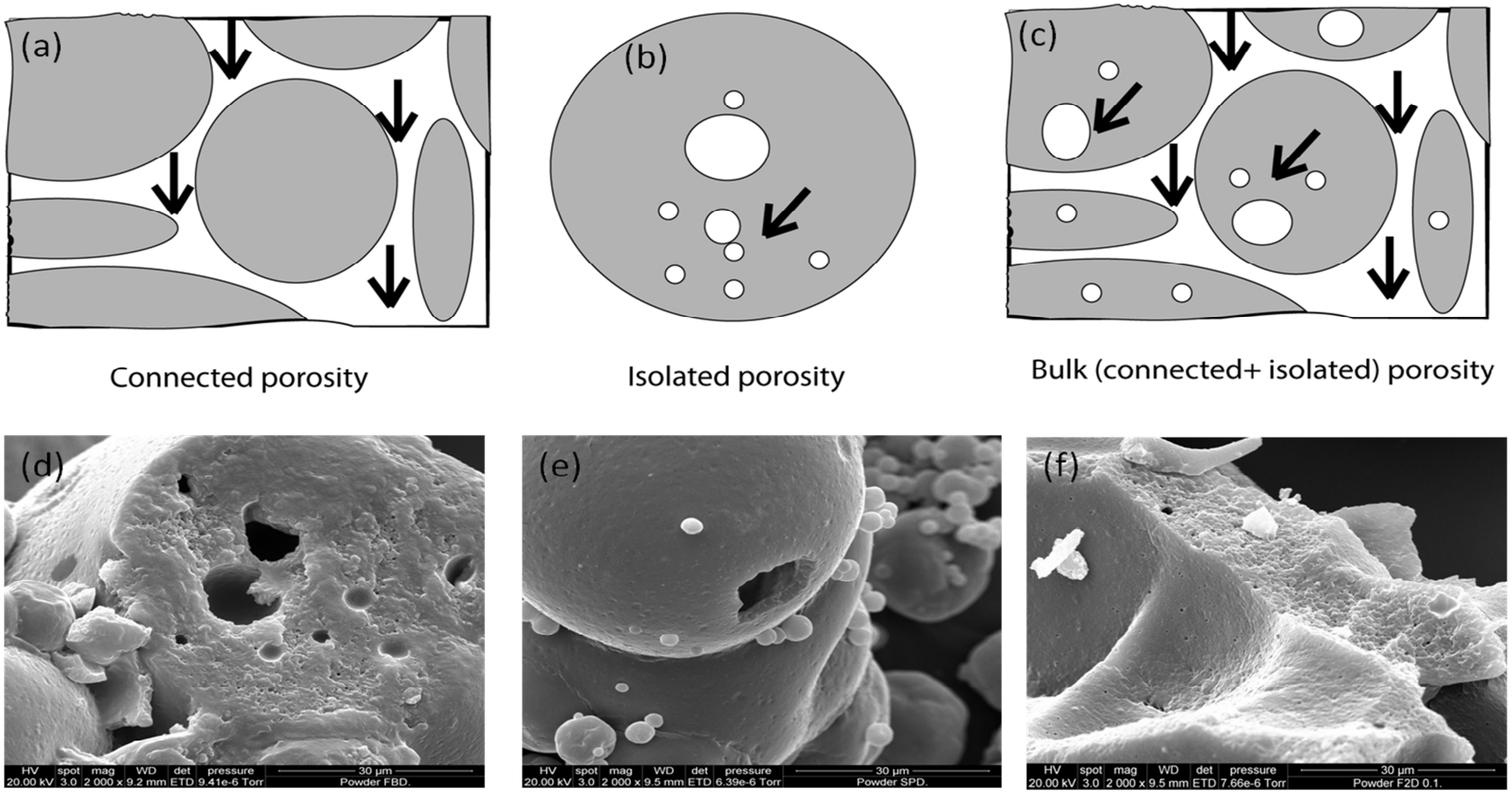

Figure 4-5 Schematic representation of the porosities of the powders: (a) connected porosity; (b) isolated porosity; (c) bulk porosity. Isolated porosity was observed in the scanning electron microscopy images of (d) the fluidized bed dried powder and (e) the spray dried powder and significant connected porosity was observed in (f) the freeze dried powder.

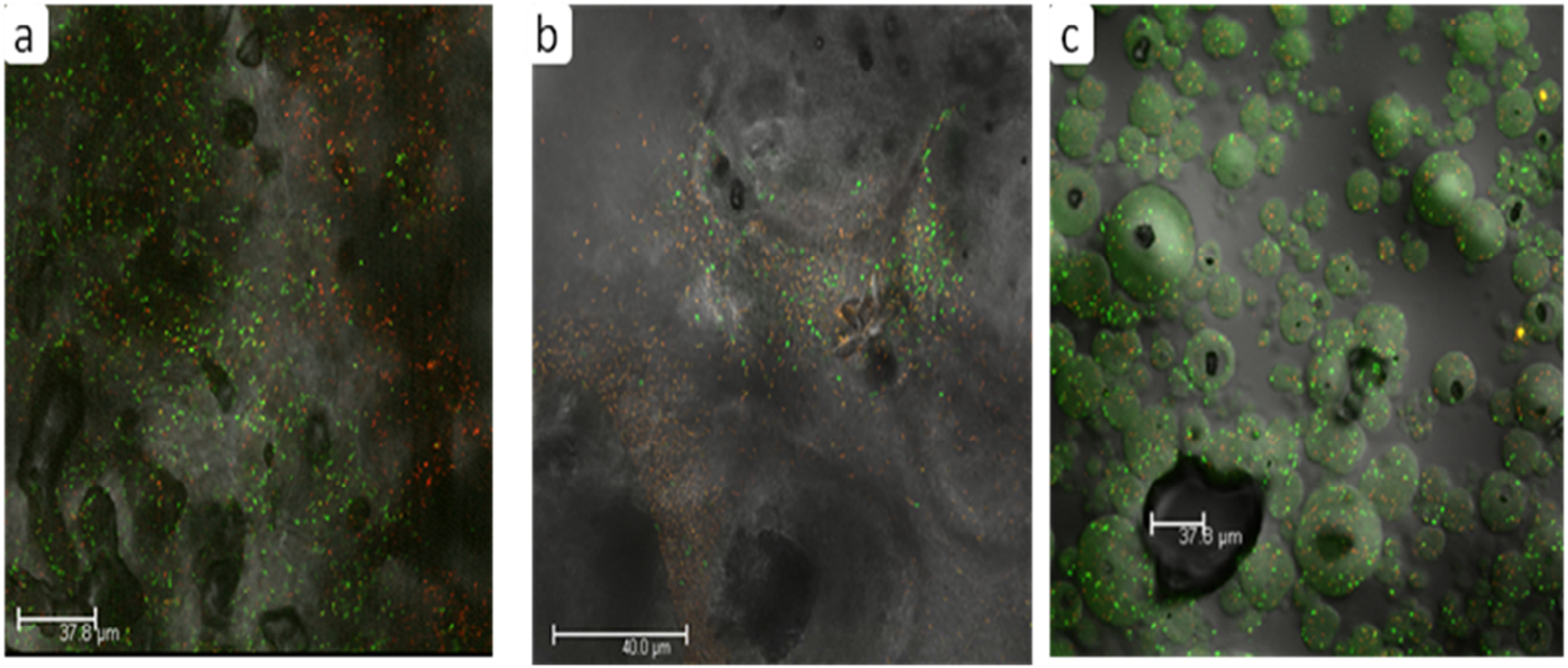

Figure 4-6 Confocal laser scanning microscopy images with differential interface contrast (DIC) and 40X magnification of a) freeze dried b) fluidized bed dried c) spray dried *Lactobacillus paracasei* 431 powders, live (green) and dead(red) indicate the live and dead bacteria.

Confocal laser scanning electron microscopy images (Figure-4-6) shows the distribution of bacteria in the powder matrix. Live and dead bacterial distribution could be observed indicated by green and red spots within the powder matrix.

4.3.4 Powder structure Characterization

4.3.4.1 X-ray diffraction

The powders prepared from whole milk containing *L. paracasei* were X-ray amorphous when freshly dried and after equilibration at a_w values of 0.11 and 0.33. However, after equilibration at 0.52 a_w, the materials showed clear Bragg peaks, indicating some crystallinity (Figure 4-7). The diffraction patterns could be assigned to β-lactose for the spray dried and freeze dried powders (Kirk et al. 2007; Shawqi Barham et al. 2006), but the major component in the fluidized bed dried powders was not clear, although it was likely to be a polymorph of lactose. 2D X-ray diffraction data is included in Appendix A-2.

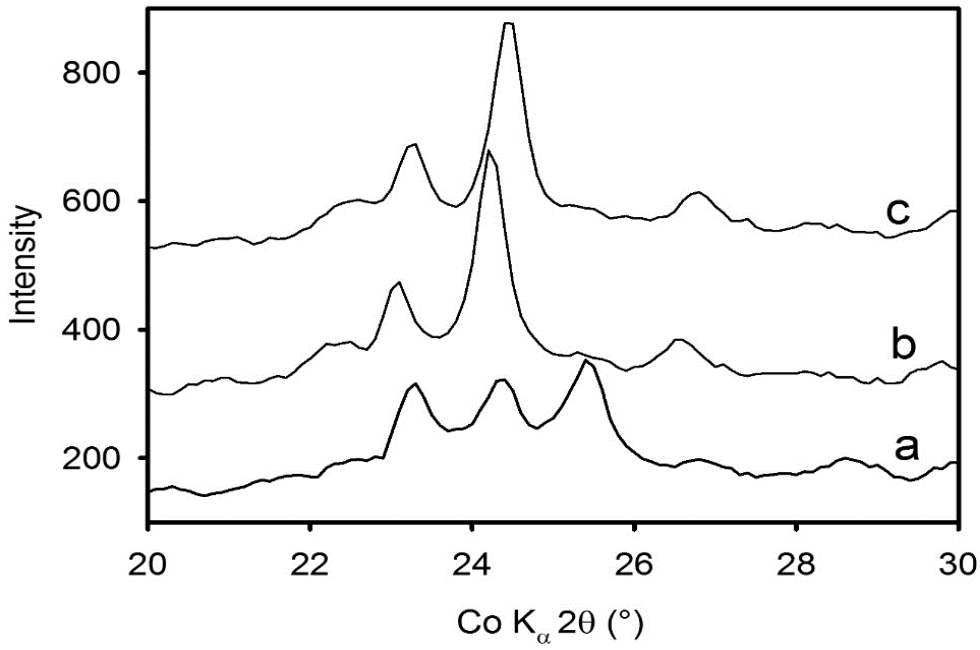

Figure 4-7 X-ray diffraction for powders containing *L. paracasei*, prepared using (a) fluidized bed drying, (b) spray drying and (c) freeze drying and equilibrated at 0.52 a_w.

4.3.4.2 Fourier transform infrared spectroscopy

The FTIR spectra of the powders prepared from whole milk containing *L. paracasei* were used to identify the major components, i.e. fat, protein, lactose and water. PCA was also used to compare the complete FTIR spectra from each of the moisture-equilibrated powders. This method looks for statistical differences between the spectra and separates them based, not on prior knowledge of the chemical significance of the spectral components, but on purely statistical criteria. For this analysis, the spectrum from 750 to 3600 cm^{-1} was used, with the exclusion of the region 1900−2750 cm^{-1} (Figure 4-8).

The resulting scores plot for PC1 versus PC2 showed a clear separation (Figure 4-8 c and d). PC1 was characterized by positive loading peaks at 1036, 1071 and 1099 cm^{-1} (which were due to lactose), 1642 and 1543 cm^{-1} (from the amide I and amide II region), 1157, 1736, 2850 and 2920 cm^{-1} (characteristic of milk fat) (Islam and Langrish, 2010; Lei et al. 2010) and 1643 and 3404 cm^{-1} (due to OH stretching). PC2 showed positive loadings for α-lactose monohydrate (peaks at 875, 896 and 916 cm^{-1}) (Kirk et al. 2007). α-Lactose monohydrate crystal patterns have previously been observed in the forced crystallization of fluidized bed dried milk powder (Yazdanpanah and Langrish, 2011). Negative PC2 loadings mainly arose from peaks representing β-lactose at 834, 873, 889 and 942 cm^{-1} (Kirk et al. 2007) and fat at 1172, 1472, 1748, 2850 and 2920 cm^{-1} (Lei et al. 2010) (Table 4-3).

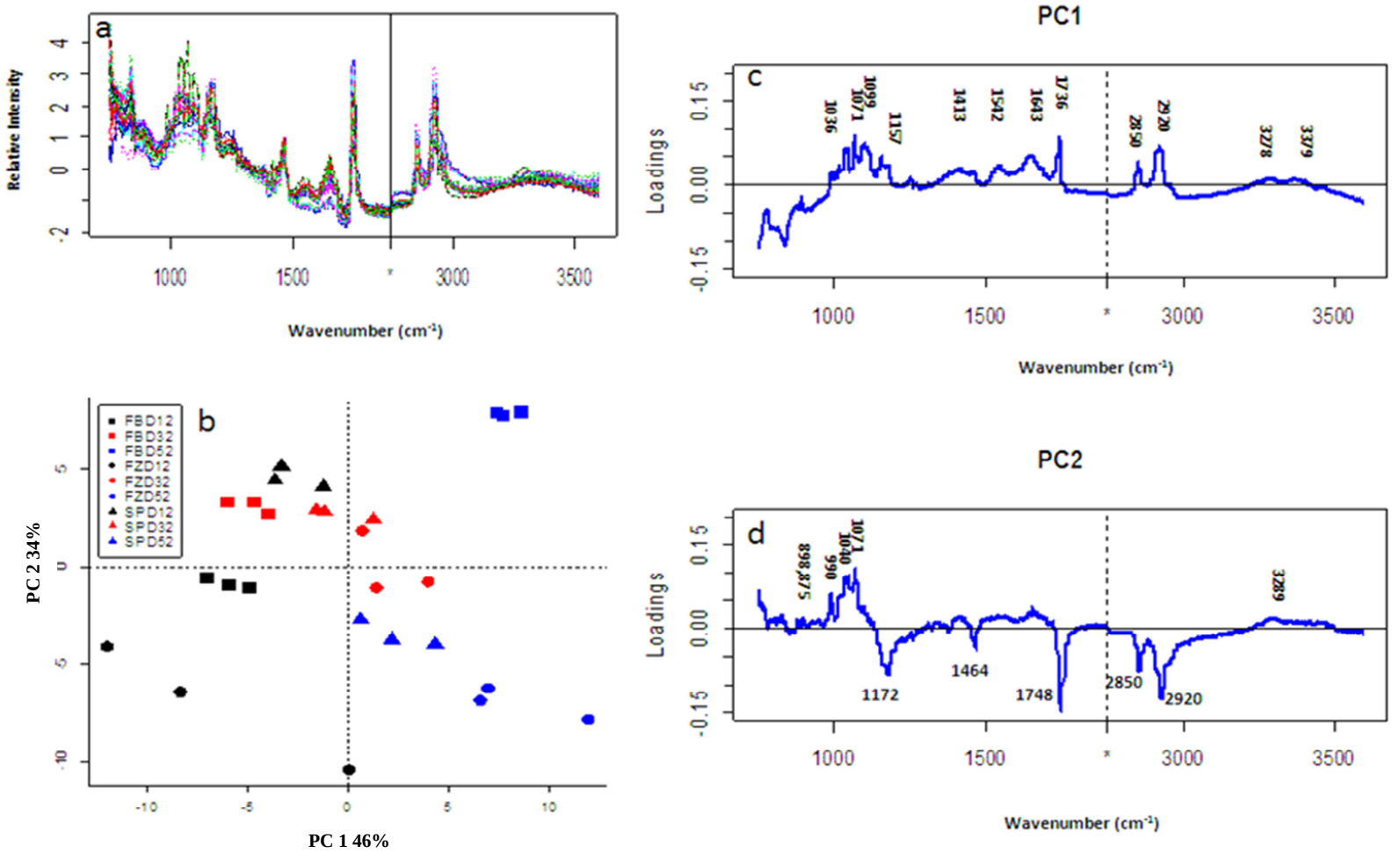

Figure 4-8 Infrared spectrum of freeze dried, spray dried and fluidized bed dried bacterial powder stored at 0.11, 0.33 and 0.52 a_w after SNV pre-processing b) Principal component analysis of FT-IR spectrum of freeze dried (circle), fluidized bed dried (square), and spray dried (triangle) *L. paracasei* powders after 1 month storage at 25°C and controlled water activity conditions of 0.11 a_w (black), 0.33 a_w (red) and 0.52 a_w (blue). **c)** PC 1 loading plot d**)** PC 2 loading plot

Table 4-3 Peaks found in the FT-IR spectra and the associated assignments to vibrational modes and to PC1 and PC2.

Peak position PC1/PC2	Peak position / cm^{-1}	Reference position / cm^{-1}	Group	Vibration mode	Attribution	Reference
+/-	2920	2920-2930	CH2	Stretching anti-symmetrically	Fat	(Kirk et al. 2007; Lei et al. 2010)
+/-	2850	2850-2855	CH2	Stretching symmetrically	Fat	(Kirk et al. 2007; Lei et al. 2010)
+/-	1736/1748	1736-1745	C=O	Stretching	Fat	(Kirk et al. 2007; Lei et al. 2010)
+/NA	1643	1640-1660	C=O	Stretching	Protein (Amide I)	(Kirk et al. 2007; Lei et al. 2010)
+/NA	1542	1541-1547	N-H	Bending	Protein (Amide II)	(Kirk et al. 2007; Lei et al. 2010)
+/NA	1413					

NA/-	1464					
+/-	1157/1172	1157-1160	C-O	Stretching	Fat	(Kirk et al. 2007; Lei et al. 2010)
+/+	1036,1071,1099/1040,1071	1030-1150	C-O,C-C, C-O-C	Stretching	Carbohydrate	(Kirk et al. 2007; Lei et al. 2010)
NA/+	990	800-1000	Carbohydrate ring	Ring vibration	Carbohydrate	(Kirk et al. 2007; Lei et al. 2010)
NA/+	903,875	875,900			Alpha lactose monohydrate	(Kirk et al. 2007; Lei et al. 2010)
+/+	3278,3379/3289	3404	OH	Streching	Water	(Kirk et al. 2007; Lei et al. 2010)

The PCA analysis (Figure 4-8) showed different behaviors with moisture uptake for the three powders prepared by the three different drying methods. All the powders stored at 0.11 a_w were in the negative PC1 space, whereas the powders stored at 0.52 a_w were in the positive PC1 space. The separating factor between the powders stored at lower and higher water activities was the protein hydration in the amide I and II region, the OH stretching and the fat mobility. Fluidized bed dried powder stored at 0.33 a_w was in the negative PC1 space whereas the spray dried and freeze dried powders stored at 0.33 a_w were in the positive PC1 space. This suggests that there was more water and greater protein hydration in the spray dried and freeze dried powders. PC2 separated the fluidized bed dried powder stored at 0.52 a_w from the spray dried and freeze dried powders stored at 0.52 a_w, based on the lactose crystal form and the fat mobility. The fluidized bed dried powder had a large amount of α-lactose monohydrate, whereas the spray dried and freeze dried powders possessed mainly β-lactose, as previously observed by X-ray diffraction. Fat mobility, characterized by $–CH_2$ stretching, was greater in the spray dried and freeze dried powders than in the fluidized bed dried powder. The higher molecular mobility in the spray dried and freeze dried powders may enable more rapid transport of oxygen, leading to membrane lipid oxidation and consequently bacterial death.

4.3.4.3 Raman spectroscopy

Powders with higher water activity have always been found to be associated with increased bacterial death. However, the presence of high amount of water in the matrix also creates interferences, which poses difficulty to study spectrum as in case of FT-IR spectroscopy. However, Raman spectroscopy provided the added advantage to study the material without interfering water signals. In the first part, spectroscopic analysis of

encapsulated bacteria and bacteria without the matrix was carried out. The spectra of the bacteria in the initial investigation were poor even with relatively long acquisition times. This result made it impossible to look into the bacteria without the matrix. Therefore, the study was focused on the changes in the matrix stabilized bacterial sample.

The principal component analysis (PCA) of the stabilized bacterial samples stored at controlled water activity conditions of a_w 0.11, 0.33 and 0.52 was carried out in the spectral regions of interest 3100-2700 cm^{-1} and 1770-330 cm^{-1} (Fig 4-9 b). PC1 accounts for 63 % of total variance characterized by high positive loading corresponding to peaks at 358, 478, 853, 873, 1017, 1086 cm-1, 1127 (lactose) and 2857 and 2885 cm^{-1} (fat) (Figure 4-9 c) (Table 4-4). The crystallization of lactose (Kirk et al. 2007) along with the increased mobility in fat (determined by the ratio of spectral intensity at 2850/2885 (Forrest, 1978)). The crystallization of lactose was found to be the major contributing factor in PC1 separation. PC2 which accounted for 17% of the variation characterized by positive loading corresponding to rigid fat, calculated from the ratio of spectral intensity at 2850/2885 (Forrest, 1978) and characterized by major peak at 360cm^{-1} corresponding to alpha lactose monohydrate (Kirk et al. 2007) (Figure 4-9d). This result is consistent with our previous observation using FT-IR spectroscopy where an increase in the C-H stretching was also noted. This result further tallied with the previous observation using XRD and FT-IR spectroscopy, where crystallization was noted at higher water activity. An increased mobility of fat was observed at higher water activity conditions. Fluidized bed drying technique was found to have rigid structure compared to the spray and freeze dried powders.

Table 4-4 Raman vibrational peaks and the associated assignments to PC1 and PC2

+/+	358/358	357	α-Lactose monohydrate and β-Lactose	(Kirk et al. 2007)
+/NA	478/	475	α-Lactose monohydrate	(Kirk et al. 2007)
+/NA	853	851	α-Lactose monohydrate	(Kirk et al. 2007)
+/-	873/870	876	α-Lactose monohydrate and β-Lactose	(Kirk et al. 2007)
+/NA	1017	1018	α-Lactose monohydrate and β-Lactose	(Kirk et al. 2007)
+/-	1086/1084	1086	α-Lactose monohydrate	(Kirk et al. 2007)
+/NA	1127	1126	C-C bond stretch (milk fat)	(Forrest, 1978)
+/NA	1263	1265	Amide III (casein unordered structure)	(Gallier et al. 2011)
+/NA	1270	1270	C=C cis unsaturation C-H in-plane bending of	(Gallier et al.

			ethylene groups	2011)
+/NA	1327	1303	PC, PI and PS	(Gallier et al. 2011)
+/NA	1441	1443	Saturated fatty acid, δ(CH2) scissoring (cholesterol,PC, PI and PS)	(Gallier,2011)
+/+	2857/2850	2850	C-H stretch (milk fat)	(Forrest, 1978)
+/+	2885/2884	2885	C-H anti symm stretch C-H symm. stretch (milk fat)	(Forrest, 1978)

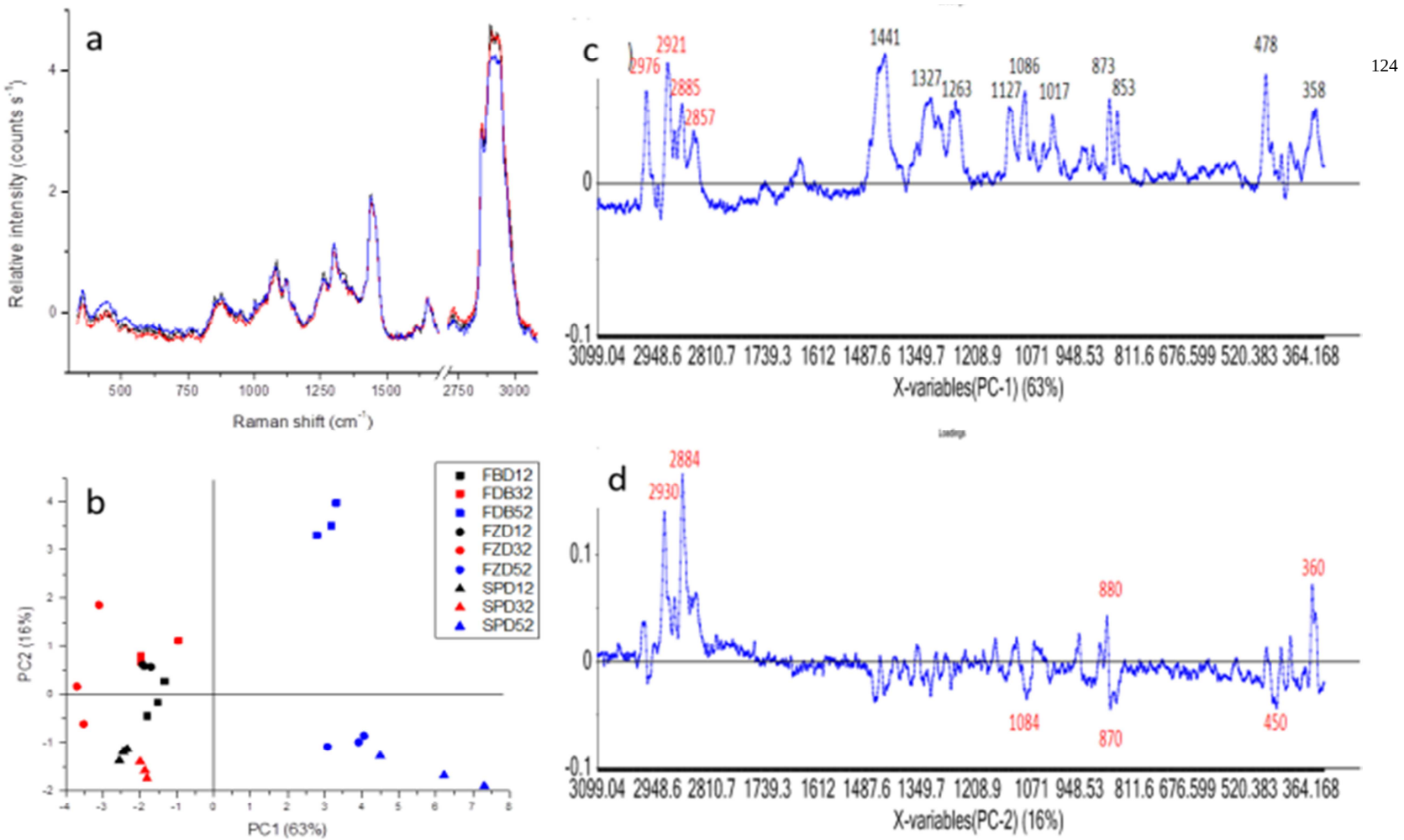

Figure 4-9 a) Raman spectrum of freeze dried, spray dried and fluidized bed dried bacterial powder stored at 0.11, 0.33 and 0.52 a_w after SNV pre-processing b) Principal component analysis of Raman spectrum of freeze dried (circle), fluidized bed dried (square), and spray dried (triangle) *Lactobacillus paracasei* powders at 0.11 a_w (black), 0.33 a_w (red) and 0.52 (blue) a_w after 1 month storage at 25°C c) PC 1 loading plot d) PC 2 loading plot

4.3.4.4 Nuclear Magnetic Resonance (NMR) spectroscopy

Molecular mobility has always been associated with bacterial death, a possible reason being the increased molecular mobility increases the rate of oxidative reactions (reactions in liquid state are faster as compared to solid state reactions). The ^{13}C solid state NMR spectrum is very useful technique to study molecular mobility. It reflects the fact that every molecule has a magnetic environment which repeats regularly so as to produce a rather narrow dispersion of chemical shifts. On the contrary, in the presence of molecular disorder, the dispersion is larger due to the large number of environments and moreover variable with time. Thus a drastic decrease of the intensity of NMR signal under this condition is to be attributed to the loss of molecular order. Typical NMR spectrum of freeze dried, spray dried and fluidized bed dried powder is shown in Fig 4.10. The NMR result suggests the fluidized bed dried powder to be more stable at higher a_w storage condition. The presence of high amount of fat in the samples causes analysis of the samples by NMR difficult. Peaks at 79, 82, and 99, 104 are indicative of beta lactose (Kirk et al. 2007), peaks at 62 and 72 ppm indicate the presence of α-lactose monohydrate. Both spray dried and freeze dried powders showed peaks at 104 regions, suggesting possible β-lactose form as seen in FT-IR results. There have been overlapping lactose peak (Kirk et al. 2007) and fat peak (Scano et al. 2011) in case WMP embedding matrix making interpretation of NMR data difficult. List of ^{13}C-NMR peaks attributed to milk fat region is given in Appendix Table A3. PLS-DA shows separation between the samples stored at 0.11 a_w and 0.52 a_w (Appendix Figure A3) attributed to mobility in fat region.

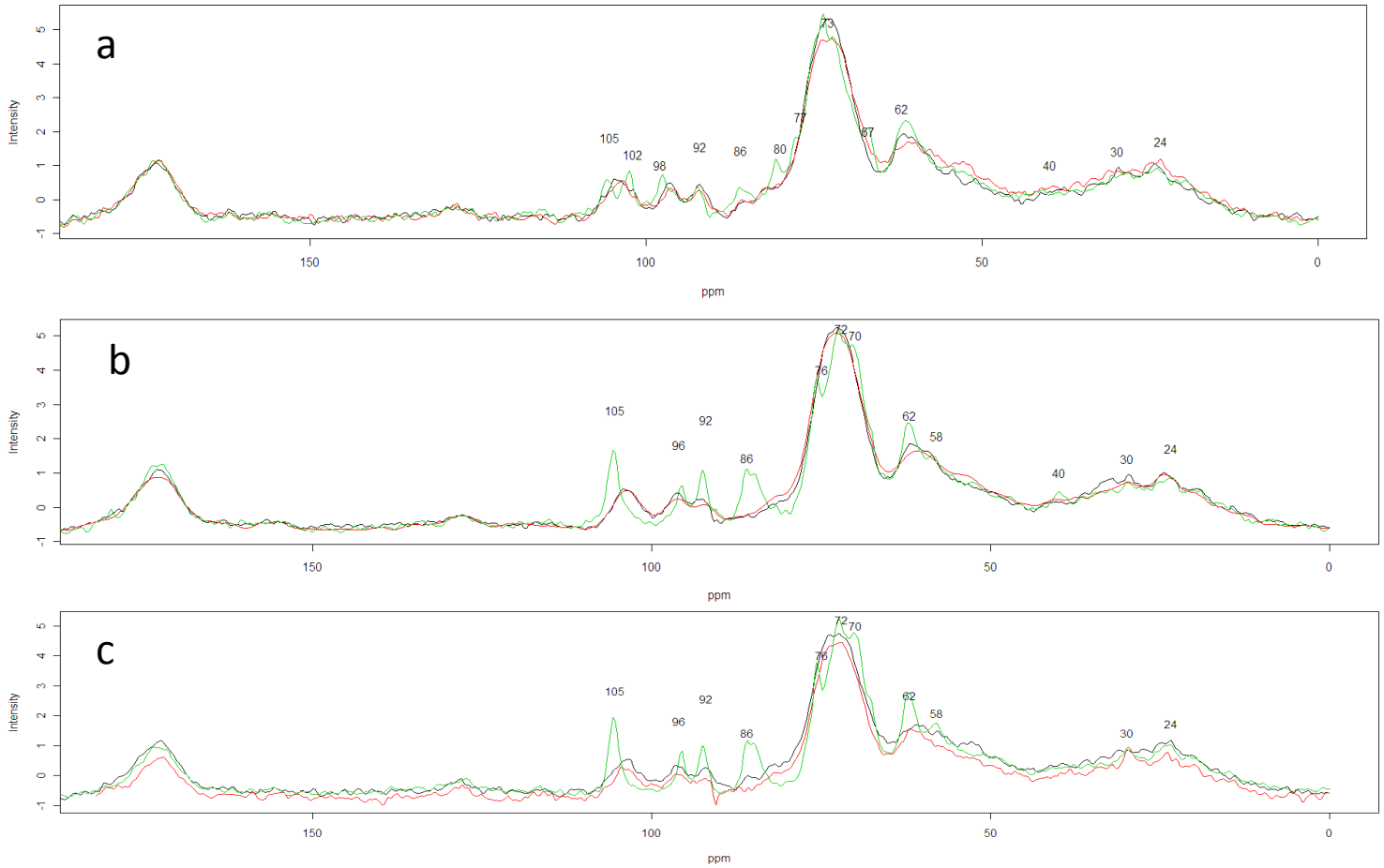

Figure 4-10 ^{13}C CP MAS spectrum of a) fluidized bed dried b) spray dried c) freeze dried *Lactobacillus paracasei* 431 powders at 0.11 (brown), 0.33 (red) and 0.52 (green) a_w after 1 month storage at 25°C.

4.3.4.5 Thermogravimetric analysis/ differential scanning calorimetry (TGA/DSC)

The previous finding of the importance of water being responsible for bacterial death during long term storage, made it essential to study the characteristics of surface water, water of crystallisation and the nature of the lactose crystals. The TGA/DSC (thermo-gravimetric analysis/ differential scanning calorimetry) technique is useful for providing such information. PCA score plots of the TGA/DSC (Figure 4-11, 4-12) agreed with the XRD result and a clear separation was observed between the higher and lower humidity samples, on the basis of crystallization.

The DSC results showed melting peaks of crystalline lactose around 200°C, which did not correspond to pure α- or β-lactose crystals (222 and 242°C); these peaks may have arisen possibly due to the effects of proteins. This observation is consistent with the previous report of similar DSC curves (lactose melting at 200 °C in β-lactoglobulin: lactose powders in 30:70 and 40:60 ratio (Thomas et al. 2004). The profile of the bound water/surface water was observed to be different during storage at controlled water activity conditions of 0.52 a_w, when compared to storage at lower water activity of 0.11 a_w and 0.33 a_w. The TGA/ DSC results are in line with the XRD results.

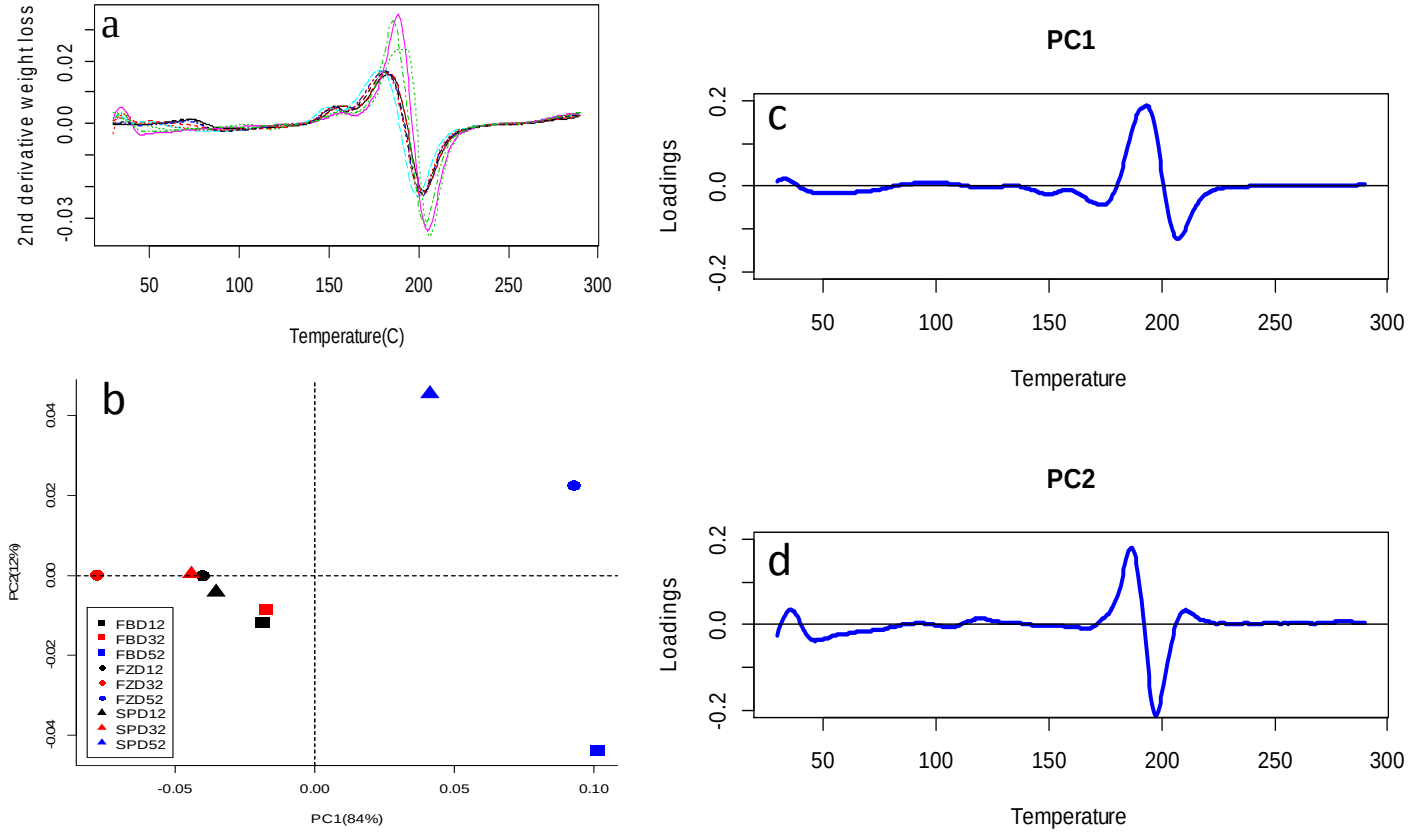

Figure 4-11 (a) Second derivative thermogravimetric analysis (TGA) of freeze dried, spray dried and fluidized bed dried bacterial powder stored at 0.11, 0.33 and 0.52 a_w after SNV pre-processing b) Principal component analysis of thermogravimetric analysis (TGA) of freeze dried (circle), fluidized bed dried (square), and spray dried (triangle) *Lactobacillus paracasei* powders at 0.11 a_w (black), 0.33 a_w (red) and 0.52 (blue) a_w after 1 month storage at 25°C c) PC 1 loading plot d) PC 2 loading plot

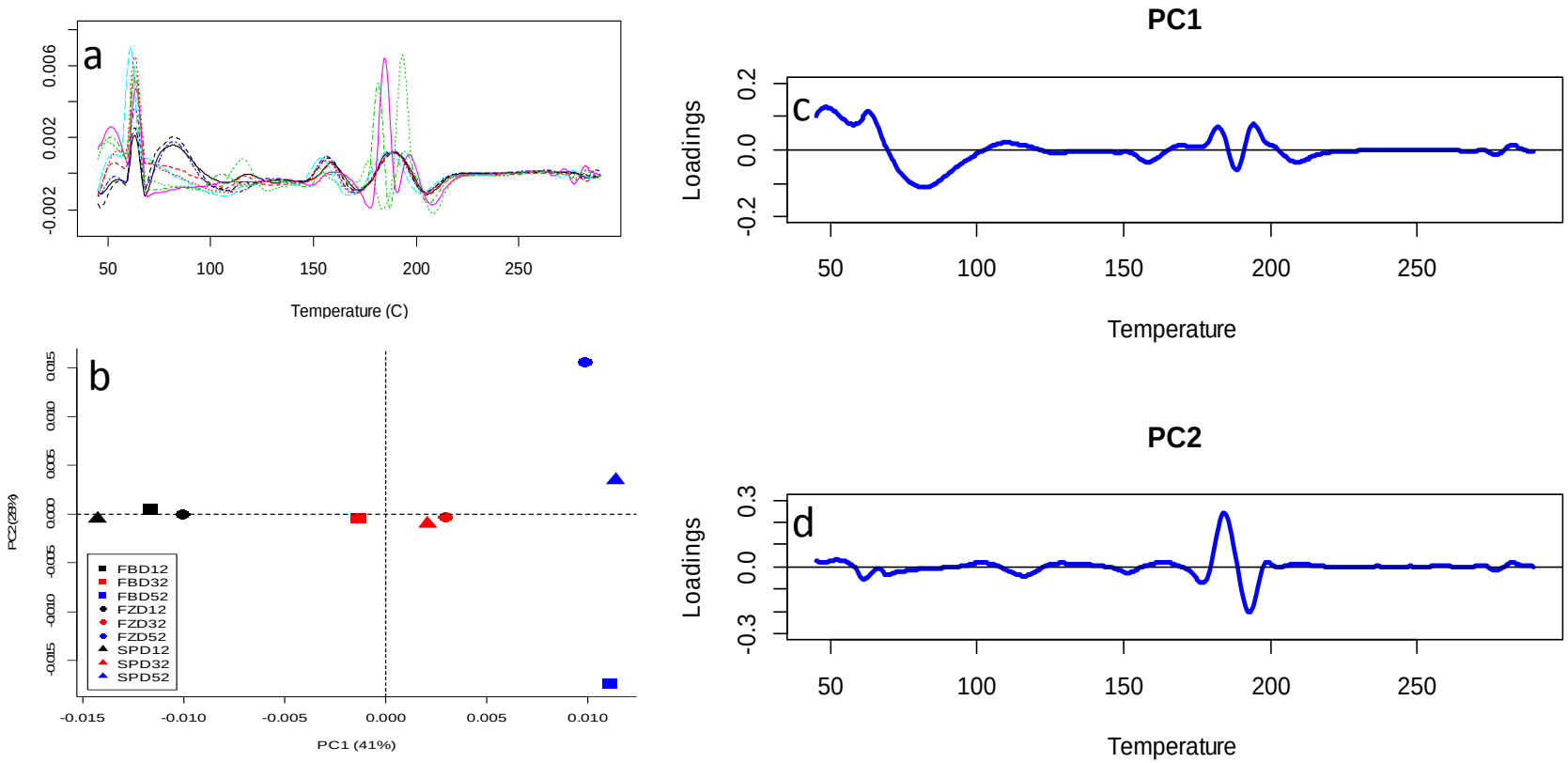

Figure 4-12 Second derivative heat flow of freeze dried, spray dried and fluidized bed dried bacterial powder stored at 0.11, 0.33 and 0.52 a_w after SNV pre-processing b) Principal component analysis of heat flow of freeze dried (circle), fluidized bed dried (square), and spray dried (triangle) *Lactobacillus paracasei* powders at 0.11 a_w (black), 0.33 a_w (red) and 0.52 (blue) a_w after 1 month storage at 25°C c) PC 1 loading plot d) PC 2 loading plot

4.4 Conclusions

It was shown that, of the three drying methods used for stabilizing *L. paracasei* bacteria in a whole milk powder matrix, fluidized bed drying provided better protection to the bacteria during storage under ambient conditions. This was attributed to the lower porosity and the larger agglomerate size, which reduced the absorption of water. The lower absorption of water resulted in the maintenance of a more rigid structure, which may have limited molecular transport, particularly of oxygen. This probably resulted in a lower rate of bacterial death. Using fluidized bed drying, it may be possible to produce dried probiotics in a milk powder matrix that have adequate storage life to provide health benefits to consumers.Effect of manganese accumulation on the viability of probiotic *Lactobacillus paracasei*

Chapter 5 Effect of manganese accumulation on the viability of probiotic *Lactobacillus paracasei*

5.1 Introduction

The work reported in Chapter 4 showed that, using fluidized-bed drying, it is possible to produce dried probiotics in a milk powder matrix that retain bacterial viability during storage. The protective effect of the fluidized-bed drying was attributed to the lower porosity and the larger agglomerate size of the powder particles. After gaining insights into the roles of matrix composition, structure and the drying process on the viability of probiotics, it was of interest to study aspects of cell physiology that may further improve the viability of probiotic lactobacilli during storage.

One possible means of increasing the shelf life of *Lactobacillus paracasei* is to incorporate manganese into the bacterium. It is believed that manganese plays a protective role in cells. For example, the radiation-resistant bacterium *Deinococcus radiodurans* features high cellular levels of manganese, which have been attributed to the remarkable survivability of this microorganism (Daly et al. 2004). Manganese has also been found to play an anti-oxidative role in the nematode *Caenorhabditis elegans* (Lin et al. 2006). It has been observed that *Lactobacillus plantarum* accumulates large amounts of inorganic manganese (30–35 mM), which has been associated with the activity of superoxide dismutase, thereby helping the bacterium to survive under harsh environmental conditions and protecting it against oxidative damage (Archibald and Fridovich, 1981a, 1981b; Barnese et al. 2008, 2012; Daly et al. 2004). It was therefore of interest to study whether or not the ability of *L. paracasei* to maintain viability in a milk powder matrix during storage is enhanced by the accumulation of manganese.

Modification of the growth phase and the cell harvesting conditions have been investigated to enhance the stress tolerance level and to improve the robustness of probiotic cultures, as indicated in the literature review (Chapter 2). Therefore, optimization of the growth phase, the medium and the cell harvesting conditions may be worthy of further exploration to improve the shelf life of bacteria. The stress resistance of a bacterial culture is dependent on the growth phase. For instance, bacteria that enter the stationary growth phase develop a general stress resistance. The increased stress response of stationary phase cells, compared with bacteria in the log phase, has previously been correlated with carbon starvation and the exhaustion of available food resources; these conditions trigger responses that allow cell survival (Van de Guchte et al. 2002). For example, probiotic *Lactobacillus rhamnosus* spray-dried powders retained high viability (over 50% survival; 2.9 x 10^9 CFU/g) when produced from stationary phase cells, whereas the same probiotic bacterial powders made from early log phase cultures exhibited only 14% survival (Corcoran et al. 2004). These findings led researchers to believe that improved bacterial viability during storage may be achieved by utilizing bacteria in the stationary phase. The scope of the current study was therefore widened to determine whether the increased stress resistance of stationary phase cells is related in any way to the level of manganese accumulation in these cells. As manganese has the ability to scavenge free radicals and to provide protection against oxidative conditions, any difference in the level of manganese ions may explain why stationary phase cells are more robust than early log phase cells. Past studies have focused on the changes in membrane fluidity, with respect to changes in the growth medium and the growth conditions, as being responsible for loss of viability (Yao et al. 2008). However, the focus of the work described in this chapter was to determine changes in the concentration gradient of metal ions during different stages of growth,

growth in manganese-rich and manganese-deficient media and their effects on bacterial viability during storage.

To ascertain the accumulation of manganese in cells, freeze-dried bacterial cells of *L. paracasei* were studied using energy-dispersive X-ray spectroscopy (EDS) and the accumulation was also established using inductively coupled plasma–mass spectrometry (ICP–MS). Subsequently, sections containing embedded bacteria were examined using scanning electron microscopy (SEM) to locate electron-dense deposits of the bacteria. These electron-dense spots were further resolved using electron energy loss spectroscopy (EELS) and EDS mapping. As neither of these techniques was able to resolve the electron-dense particles observed using SEM, possibly because of the very small particle size (less than 20 nm), it was thought that an X-ray fluorescence microscopy (XFM) technique would allow unequivocal identification of manganese in these deposits (and elsewhere in the cell). Synchrotron XFM is a viable tool for the non-invasive characterization of hydrated cells, with a spatial resolution of about 150 nm, and would be ideal for studying manganese in bacteria *in vivo*. Therefore, synchrotron XFM was employed to determine changes in the elemental composition of *L. paracasei* ATCC 55544, during growth (manganese-rich medium versus manganese-deficient medium) and as a function of the physiological growth state (early log phase versus stationary phase). Further, the changes in cell viability during storage were assessed by embedding the bacteria using fluidized-bed drying and storing the milk powder matrix.

5.2 Materials and methods

5.2.1 Bacterial growth conditions

A fresh culture of *L. paracasei* was grown in MRS broth and was harvested after 18 h of growth at 37ºC, under microaerophilic conditions. The harvested bacteria were freeze dried (using parameters as outlined in Section 4.2.2.2).

For the XFM study, the bacteria were grown in MRS medium and were harvested after 4 and 18 h (as outlined in Section 3.3.1). The bacteria were also grown in reconstituted MRS broth (without added manganese) and were harvested after 18 h. Typical composition of MRS broth and reconstituted MRS broth without added manganese is shown in Appendix Table A1.

5.2.2 Embedding of bacteria

The bacteria were embedded in a milk powder matrix using a fluidized-bed drying technique (as described in Section 4.3.2.3) and were stored at a water activity of 0.33 at 25°C for a period of 15 days.

5.2.3 Inductively coupled plasma–mass spectrometry and optical emission spectroscopy

Inductively coupled plasma–mass spectrometry (ICP−MS) and optical emission spectroscopy (OES) were used for elemental analysis. Freeze-dried bacteria and milk powder samples were digested in acid (hydrochloric acid/nitric acid mix, HCl:HNO_3 = 3:1) at 80°C for 1 h, cooled to room temperature, diluted and then filtered. The manganese content was determined at Hill Laboratories (Hamilton, New Zealand) using either a Sciex Elan-DRCII system or a Sciex Elan-6100DRC Plus ICP−MS system

(Perkin Elmer Life and Analytical Sciences, USA). The analysis method used was APHA 3125B (metals by ICP−MS). Phosphorus was analyzed using ICP−OES on a Thermo IRIS Intrepid XDL Radial system (Thermo Fisher Scientific, USA). The results are expressed as mg/kg – equivalent to parts per billion.

5.2.4 Scanning electron microscopy

Cells were placed on a glass coverslip and were fixed in 2.5% glutaraldehyde solution for 6 h at 4°C; they were then washed with Milli-Q water and air dried on the coverslip. The coverslip was then crushed and sprinkled on to double-sided tape on an aluminium SEM specimen stub; the loose particles were blown off with a hand air puffer and the specimen stub was viewed using an FEI Quanta 200 scanning electron microscope. Digital images were saved at the magnifications required.

5.2.5 Energy-dispersive X-ray spectroscopy

An FEI Quanta 200 system (SEM combined with EDS) was used to provide an experimental determination of the elemental composition of a specimen. To record the elemental composition, an electron beam was scanned across the specimen.

5.2.6 X-ray fluorescence microscopy analysis at Australian Synchrotron

A synchrotron is a machine that accelerates charged particles such as electrons to extremely high energies, creating an electron beam that travels at almost the speed of light. This is done by creating strong magnetic and electric fields and simultaneously increasing (synchronously ramping) the strength of the fields. Powerful electromagnets are used to focus and steer the beam inside a ring-shaped vacuum chamber, which minimizes collisions with air molecules and allows storage of the beam at high energy levels for many hours.

Figure 5-1 Photograph of Australian Synchrotron storage ring, in which electrons travel at close to the speed of light (Australian Synchrotron, 2012).

Synchrotrons were initially used only in the field of particle physics, allowing scientists to study collisions between subatomic particles with higher and higher energies. It was observed that, when high energy electrons are forced to travel in a circular orbit, they release extremely intense radiation – or 'synchrotron light'. Synchrotron light has many useful properties and can be filtered and directed down 'beamlines' for use in a wide range of non-destructive, high resolution, rapid, *in situ*, real-time imaging and analysis techniques. Because of the usefulness of synchrotron light, many accelerators today now exist solely to generate light for scientific experiments. These facilities that are dedicated to the production and use of synchrotron radiation are known as synchrotron light sources or synchrotron radiation facilities, and are commonly referred to as 'synchrotrons' by users.

Synchrotron radiation has become a premier research tool for the study of matter in recent years. First-generation synchrotron light sources were basically beamlines that were built on to existing facilities and were operated primarily for high energy or nuclear physics. Second-generation synchrotron light sources were dedicated to the production of synchrotron radiation and employed electron storage rings to harness the synchrotron light. Current (third-generation) synchrotron light sources optimize the

intensity of the light by incorporating long straight sections into the storage ring for ‘insertion devices’ such as undulator and wiggler magnets. Wigglers create a broad but intense beam of incoherent light. Undulators create a narrower and significantly more intense beam of coherent light, with selected wavelengths, or ‘harmonics’, that can be ‘tuned’ by manipulating the magnetic field in the device. Laboratories around the world are now working to overcome the technical challenges associated with the development of fourth-generation light sources. The fourth-generation synchrotron light source will have a hard X-ray (wavelength less than 1 Å) free-electron laser based on a very long undulator in a high energy electron linear accelerator. This device will have a peak brightness that is many orders of magnitude beyond that of the third-generation sources.

Figure 5-2 Photograph of Australian Synchrotron facility (Australian Synchrotron, 2012).

To synchrotron users, the Australian Synchrotron (Fig 5.2) provides an intense source of light, ranging from infrared to hard X-rays, that is supplied at the end-stations of beamlines. The high quality of synchrotron light improves the experimental accuracy, clarity, specificity and timeliness compared with those obtained using conventional laboratory equipment. Synchrotron techniques can generate images plus elemental, structural and chemical information from diverse sample types ranging from biological materials to industrial materials. The broad range of available wavelengths allows

scientists to carry out a large number of multidisciplinary experiments from cell biology to metallomics.

Figure 5-3 Layout representing Australian Synchrotron (Australian Synchrotron, 2012).

1. Electrons are produced at the electron gun by thermionic emission (Figure 5-3) from a heated tungsten matrix cathode. Applying a 500 MHz voltage signal to the gun as it fires means that the electrons are generated in bunches 2 ns apart. The emitted electrons are then accelerated to an energy of 90 keV, by a 90 kV potential difference applied across the gun, and move into the linear accelerator.

2. The linear accelerator (or linac) accelerates the electron beam to an energy of 100 MeV over a distance of about 10 m. This involves a series of radiofrequency cavities operating at a frequency of 3 GHz. Because of the nature of the acceleration, the beam must be separated into discrete packets, or ‘bunches’, with a spacing consistent with the 3 GHz acceleration frequency of the linac. This is done at the start of the linac, using several ‘bunching’ cavities. The linac can accelerate a beam once every second. After the first metre of acceleration in the linac, the electrons are already travelling at more than 99.99% of the speed of light.

3. The booster is an electron synchrotron that is 130 m in circumference and takes the 100 MeV beam from the linac and increases its energy to 3 GeV. The booster ring contains 60 combined function (steering and focusing) electromagnets to keep the electrons inside the stainless steel vacuum chamber and a single five-cell radiofrequency cavity (operating at 500 MHz) to supply energy for acceleration. The beam is accelerated by a simultaneous ramping of magnet strength and cavity fields. Each ramping cycle takes approximately a second for a complete ramp up and down. An electron spends about half a second in the booster ring and completes over one million laps.

4. The storage ring (Figure 5-1) is the final destination for the accelerated electrons. It can hold 200 mA of stored current with a beam lifetime of over 20 h. The storage ring is 216 m in circumference and consists of 14 nearly identical sectors. Each sector consists of a 4.4 m straight section and an 11 m arc. Every arc contains two dipole ‘bending’ magnets in which synchrotron light will be produced. Most of the straight sections have room for an ‘insertion device’. The storage ring also contains a large number of quadru-pole and sextupole magnets that are used for beam focusing, chromaticity corrections and orbit corrections.

5. Individual beamlines are positioned to capture the synchrotron light given off by the storage ring. The first section of every beamline is the photon delivery system (also called the ‘beamline optics’). It incorporates filters, monochromators, mirrors, attenuators and other devices to focus and select appropriate wavelengths for particular research techniques.

6. Experiments employing synchrotron light are conducted in customized facilities called end-stations. Most of the end-stations are housed inside radiation-shielding

enclosures called ‘hutches’ to protect staff and visitors from potentially harmful X-rays. Each beamline utilizes the synchrotron light to gather data in the form of images, chemical spectra and/or scattered light. Because research scientists cannot enter the hutches during data collection, much of the equipment is controlled remotely via motors and robotic devices (Australian Synchrotron, 2012).

5.2.6.1 X-ray fluorescence microscopy

Principle: When a sample is bombarded with X-rays, the material is ionized. Ionization occurs when an atom is exposed to energy that is greater than its ionization potential, resulting in the ejection of one (or more) electron (core electron) from the atom. The removal of the electron results in an unstable electronic structure of the atom, i.e. a highly excited core hole state. The electrons in a higher orbital come down to replace the hole left behind in the lower orbital as a result of the ionization. When an electron falls from a higher orbital to a lower orbital, the falling energy is released in the form of a photon. The energy of this photon is equal to the energy difference between the two orbitals. Each element has a characteristic emitted radiation that is generally well resolved from the emitted radiations of neighbouring elements in the periodic table. The term ‘fluorescence’ here means that the absorption of X-ray radiation (ionizing radiation) results in re-emission of the radiation (in the form of a photon, with lower energy). The X-ray fluorescence facility at Australian Synchrotron (Figure 5-4) that was used for the study provided micron and sub-micron length scale images using a KB mirror microprobe and a zone-plate (ZP) nanoprobe respectively. Four different techniques are available: SXFM, scanning X-ray fluorescence microscopy; XRF-μCT, X-ray fluorescence micro-computed tomography; XANES, X-ray absorption near-edge structure – single point mode (Vortex) and mapping mode (Maia) for oxidation state fingerprinting; DPC, differential phase contrast imaging of low-Z ultrastructure. SXFM

was used in this study. A ZP nanoprobe, which can probe a spot size of around 100 nm (0.1 µm), was used rather than a KB microprobe, which has a spot size range from 2 µm x 1 µm (H x V) to 10 µm x 10 µm.

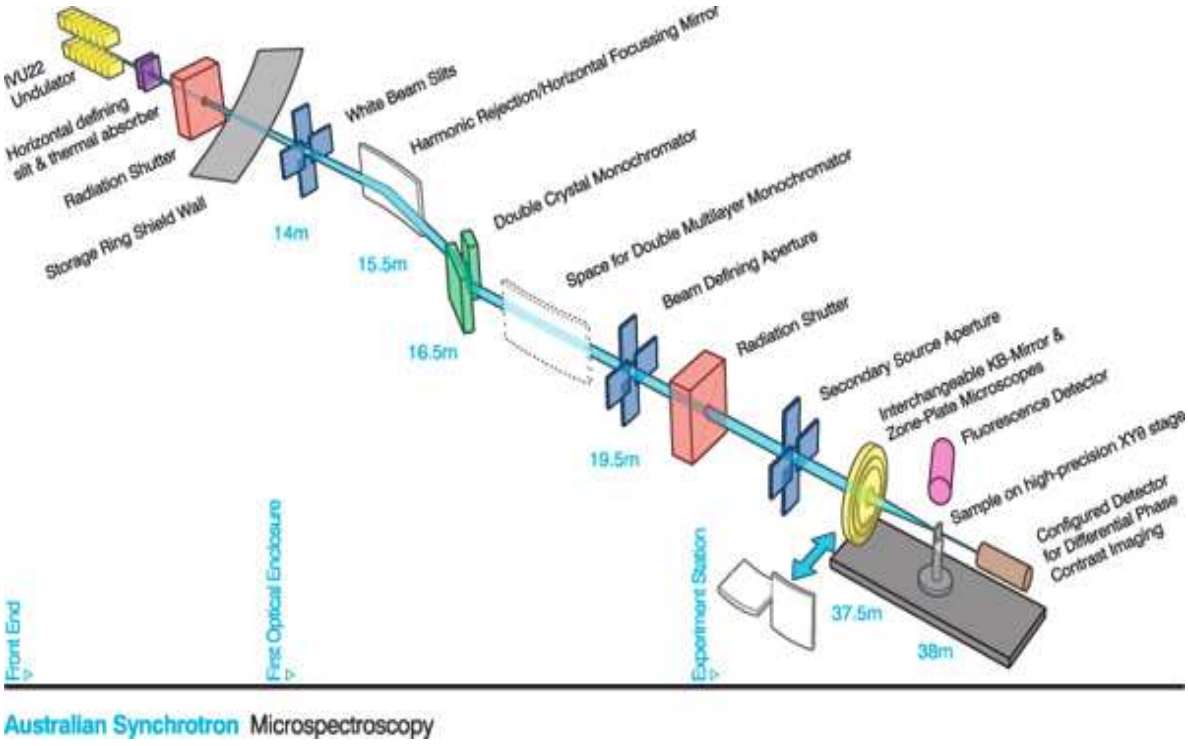

Figure 5-4 Representation of XFM at Australian Synchrotron Lightsource (Australian Synchrotron, 2012).

Two fluorescence detectors are available at Australian Synchrotron, a Vortex detector and a Maia detector. The dwell time of the Vortex detector is around 0.2–2 s/pixel, compared with the Maia detector, which has a dwell time of 0.5–50 ms/pixel. However, the Maia detector is sensitive to photons with energy above 3.3 keV [K-lines of elements heavier than calcium (Z = 20)], whereas the Vortex detector is sensitive to photons with an energy range above 1.1 keV [K-, L- or M-lines of all elements heavier than magnesium (Z = 12)]. As it was of interest to track the amount of phosphorus in the sample, together with manganese, the Vortex detector, rather than the Maia detector, was chosen for the study.

The scan duration of a sample that is being studied is governed entirely by the total number of pixels in the scan and the dwell time at each pixel. The scan area is mostly governed by the time constraint. The selection of a good substrate is a very important aspect of XFM. A general rule of thumb is that the substrate should not produce signals in excess of that expected from the specimen itself; it is generally preferred that the substrate is relatively small so that the fluorescence detector can be brought very close to the sample. Silicon nitride grids were used in this study as they come with fields of view up to 10–20 or 30 nm.

Trace metals are an important part of life sciences and understanding their changes in a biological system is important. There are several advantages to using XFM: it is possible to map more than 10 elements simultaneously; no dyes are required; XFM provides a high signal to background ratio – sub parts per million sensitivity, increasing with Z; as it is possible to study large penetration depths (> 100 μm), sectioning is not required; the incident beam is monochromatic; it is possible to choose the Z at which to

stop the excitation, e.g. it is possible to excite arsenic but not lead; because of the lower dwell time, the sample is less prone to radiation damage.

5.2.6.2 Beamline specification

Source in-vacuum undulator, n = 90, 22 mm period

Energy range 4.1–25 keV

Optimal energy range 5–12 keV

Energy resolution _E/E = 10-4

Beam properties at 10 keV	Focusing method	Zone plate
	Spot size 5 μm	100 nm
	Flux (photons/s)	10^{10}
	Flux density (photons/s/μm^2)	1 x 10^{12}

5.2.6.3 Cell harvesting and synchrotron XFM analysis

5.2.6.3.1 Sample preparation

The freshly harvested bacterial cells were washed three times in deionized water to minimize metal contamination; 10 μl of bacterial sample was suspended in deionized water, then deposited on to a silicon nitride membrane grid (Silson Ltd) and air dried in a dust-free environment for 30 min. Following drying, cells were observed under an optical microscope. The position of the cells on the grid was recorded visually by phase contrast microscopy, for subsequent positioning of the synchrotron beam. *L. paracasei* cells were distinguished from chemical precipitates based on their distinct morphology,

as observed using SEM and confocal laser scanning microscopy (CLSM). The fixed cells were analysed and imaged on the synchrotron XFM beamline at Australian Synchrotron. A ZP nanoprobe and the Vortex detector were used in this study. A beam of 10 keV X-rays was focused to a spot of 10 μm x 10 μm, using the ZP nanoprobe at the XFM beamline. The sample was scanned through the focus using the Vortex detector system, oriented at 90º to the incident beam, with a dwell time of 1.5 s/pixel. This configuration was found to image low concentrations of manganese and other elements at the cell wall, as well as the electron-dense particles.

In a separate experiment, XFM was used to study the *in situ* location of probiotic *L. paracasei* encapsulated in a dairy matrix using manganese as a tracer. XFM was also assessed for its ability to distinguish between live and dead bacteria, and was compared with CLSM.

5.2.6.3.2 Data analysis and quantification

Known elemental standards were run to quantify the amount and the gradient of metal ions in the bacterial cells (Appendix C). Data analysis involved MAPS software to fit the fluorescence data. The MAPS analysis package was used to view the data acquired by the Vortex detector. The steps involved downloading the IDL virtual machine from the following link.

http://www.ittvis.com/Login/tabid/161/Default.aspx?returnurl=%2fDownload%2fDownload.aspx%3fproduct%3dIDL

The MAPS manual and the program were then downloaded from the following link.

http://www.stefan.vogt.net/downloads.html

The MAPS software was useful for much of the data interrogation and visualization.

5.3 Results and discussion

5.3.1 Preliminary characterization of manganese accumulation in *L. paracasei*

5.3.1.1 Energy-dispersive X-ray spectroscopy

Freshly harvested bacteria grown in MRS broth were freeze dried and EDS was used to look for the presence of manganese in the bacteria grown in MRS medium. This was a preliminary study to confirm that manganese accumulated in *L. paracasei* when grown in a manganese-rich medium. As the EDS spectrum did reveal the presence of manganese (Fig. 5-5), an alternative technique, ICP–MS, was used to quantify it further. The level of manganese in freeze-dried *L. paracasei*, as measured using ICP–MS, was 1.55 wt% (15,500 mg/kg) (Table 5-1). The contribution of changes in the level of manganese to maintaining the viability of probiotics in a milk powder matrix was thought to be an important aspect for further consideration.

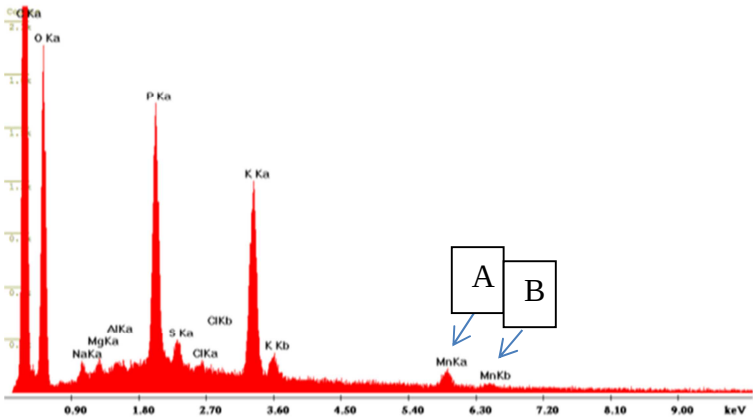

Figure 5-5 EDS of freeze-dried powder, A-manganese K-alpha emission, B-manganese-K beta emission.

Table 5-1 Elemental compositions (mg/kg) of freeze-dried *L. paracasei*, the dairy matrix [whole milk powder (WMP)] and freeze-dried bacteria embedded in the dairy matrix, determined by ICP–OES/MS

Elements	Freeze-dried *L. paracasei*	Dairy matrix (WMP)	Freeze-dried *L. paracasei* + WMP
Iron (ICP–OES)	22	< 1.3	Not analysed
Copper (ICP–MS)	10.4	0.31	Not analysed
Manganese (ICP–MS)	15,500	0.21	65–73
Phosphorus (ICP–OES)	85,000	Not analysed	Not analysed

The elemental compositions of the freeze-dried bacteria, the dairy matrix and the freeze-dried bacteria embedded in the dairy matrix revealed that the dairy matrix contained only a very small amount of manganese – 0.21 mg/kg (Table 5-1). Therefore, the majority of the signal detected in the matrix would arise from the manganese present in the bacteria within the matrix. Iron and copper were present at very low levels in the bacteria, i.e. 22 and 10.4 mg/kg respectively, compared with manganese (15,500 mg/kg). The phosphorus content in the bacteria was 85,000 mg/kg. The manganese level was diluted (65–73 mg/kg) when the bacteria were embedded in the dairy matrix, because of the small amount of bacteria that were embedded.

The size and the morphology of *L. paracasei* were observed using SEM. It was important to estimate the size and the shape of the *L. paracasei* cells, for comparison

with the bacteria as observed using XFM. From the images shown in Figure 5-6, the bacteria were estimated to be 2–3 µm in length and 0.5 µm in width.

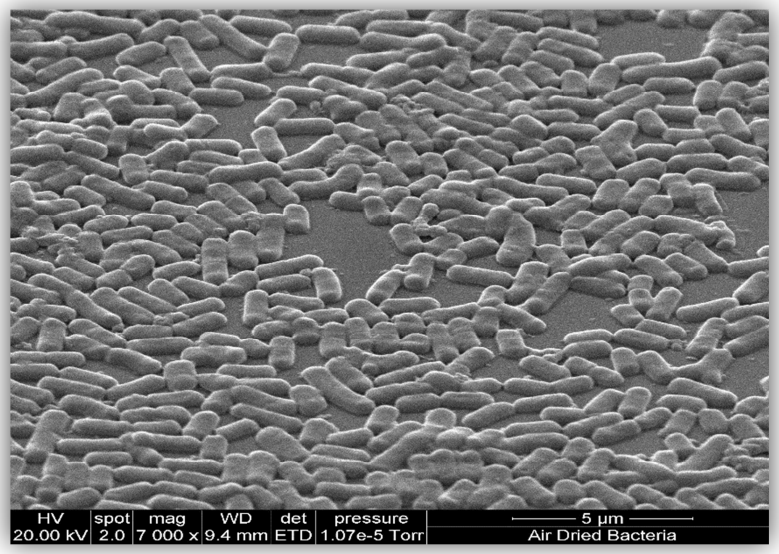

Figure 5-6 Scanning electron micrograph of air-dried *L. paracasei*.

Synchrotron XFM is a viable tool for the non-invasive characterization of hydrated cells, with a spatial resolution of about 150 nm. However, limited research using synchrotron XFM has been carried out on probiotic lactobacilli; most researchers have focused on studying multicellular microorganisms, such as animals and plants, because of the issues associated with the resolution of small bacteria. The present work was a step towards understanding the metal ion gradient in *Lactobacillus* sp. and was one of the first studies of its type, i.e. using XFM to uncover the metal ion gradient. The role of manganese in cells has been of great interest, leading to characterization of the radiation-resistant bacterium *Deinococcus radiodurans*. High cellular levels of manganese have been associated with the remarkable survivability of this microorganism (Daly et al. 2004). Manganese has also been found to play an anti-oxidative role in the nematode *Caenorhabditis elegans* (Lin et al. 2006). McColl et al. (2012), using direct *in situ* mapping by XFM, revealed that manganese and other elements are strictly maintained and enriched within specific cell types of *C. elegans*.

Previous studies have investigated the state of manganese in cells by extracting and analyzing the cell lysate (Archibald and Fridovich, 1982a). This procedure potentially changes the original state and form of the manganese. To overcome these limitations, XFM was used for *in situ* characterization of manganese and other metal ion gradient changes in bacteria that could potentially be related to bacterial viability. At the XRF facility at Australian Synchrotron, the relationship between concentration and intensity is determined by comparing with standards, and computational algorithms are used check and rectify the absorption differences between samples and standards.

Synchrotron XFM was employed to determine changes in the elemental composition of *L. paracasei* during growth (manganese-rich medium versus manganese-deficient medium) and as a function of the physiological growth state (early log phase versus stationary phase). The early log phase cells (4 h of growth) had low levels of manganese (0.0183 $\mu g/cm^2$), whereas the stationary phase cells (18 h of growth) retained relatively high levels of manganese (0.366 $\mu g/cm^2$) (Table 5.2). As expected, the bacteria grown in the manganese-deficient medium had low levels of manganese (0.0225 $\mu g/cm^2$) (Table 5.3). The estimated concentrations of phosphorus (P), potassium (K), manganese (Mn) and calcium (Ca) found in the cells are shown in Table 5-2, 5-3.

The low levels of manganese accumulation during the early log phase and on growth in a manganese-deficient medium may provide less protection to the bacteria against oxidative stress. In previous studies, early log phase harvested cells were found to provide inferior protection, compared with stationary phase cells, upon spray drying (Corcoran et al. 2004).

Table 5-2 Elemental compositions, determined by XFM, of *L. paracasei* grown in MRS medium and harvested at various stages of growth

Elements	4 h (Maximum μg/cm^2)	18 h (Maximum μg/cm^2)
P	1.23	2.86
K	1.72	1.18
Mn	0.0183	0.366
Ca	0.0699	0.131

Table 5-3 Elemental composition, determined by XFM, of *L. paracasei* grown in MRS medium without manganese

Elements	18 h (Maximum μg/cm^2)
P	2.26
K	1.62
Mn	0.0225
Ca	0.205

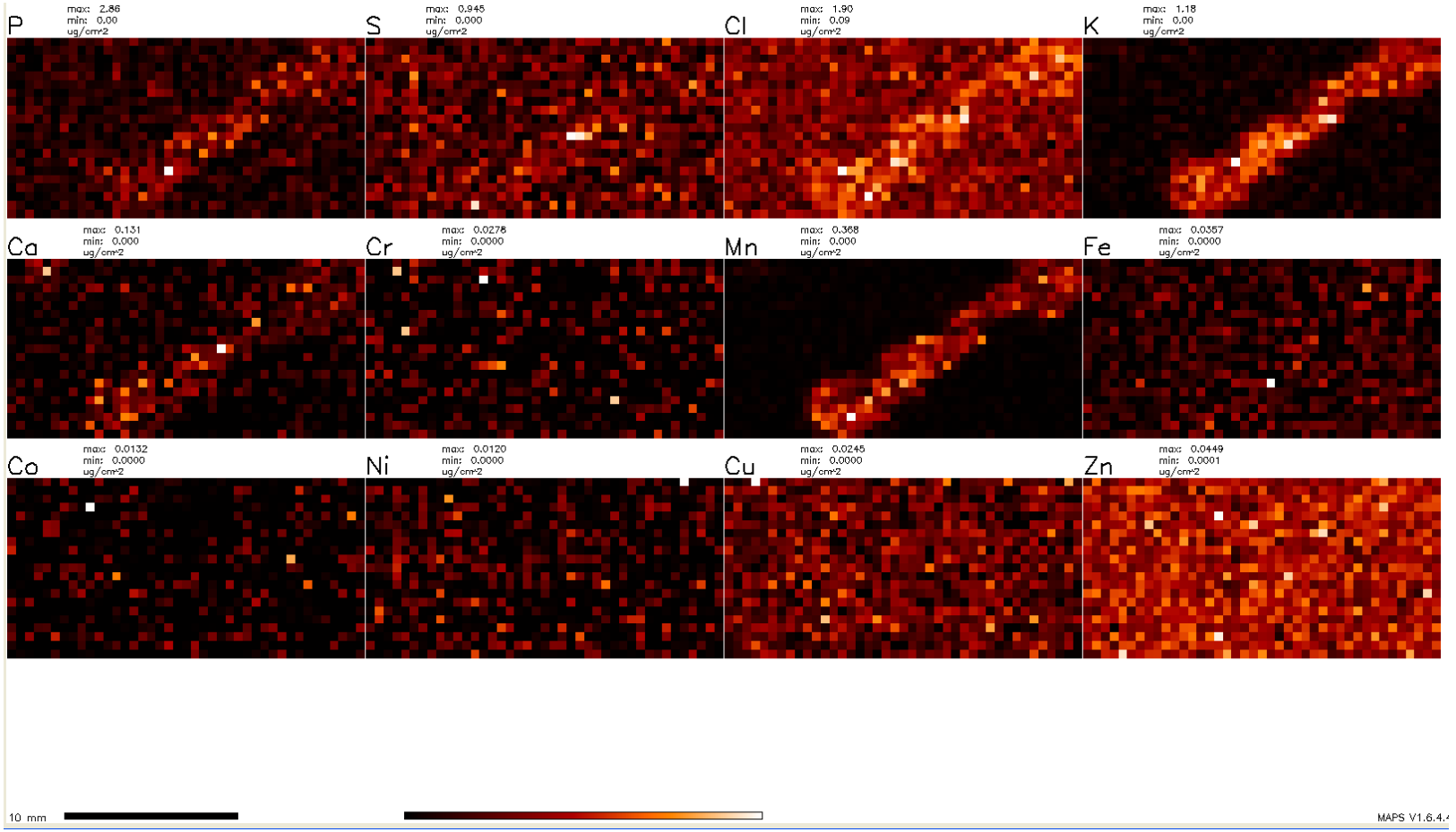

Figure 5-7 Synchrotron radiation XFM microprobe mapping of elements (phosphorus to zinc in individual channels) in a single *L. paracasei* cell grown in a manganese-rich growth medium (MRS medium). Beam spatial resolution (V × H): 0.1 µm × 0.1 µm; colour scale in counts per pixel. Manganese distribution shows distribution throughout the cell.

Figure 5-7 shows the mapping of an *L. paracasei* cell with respect to different elemental compositions (phosphorus to zinc, from left to right), when grown in MRS medium containing manganese and harvested after 18 h. A black colour in the channel represents the absence of any element, whereas a red to orange colour represents a higher proportion of the element. The manganese channel and the potassium channel were clearly separated from the background, suggesting manganese uptake by the cells, and the shape of the bacterial cells was visible. A three element co-location view of the phosphorus, manganese and potassium channels is presented in Figure 5.8. Because of the very small size of the bacteria, the elemental co-location could not be observed in much detail. However, this technique is useful in studying elemental locations within animal and plant cells.

Figure 5-9 shows the elemental mapping of the bacteria grown in MRS medium without manganese and harvested after 18 h of growth. Analysis of the images suggested that, when grown in a manganese-deficient medium, as well as lower levels of manganese, the accumulation of phosphorus was reduced (Table 5-3). Figure 5-10 shows the three element co-location view of the bacteria grown in MRS medium without manganese, harvested after 18 h of growth.

Figures 5-11 and 5-12 show elemental mapping of the bacteria grown in MRS medium containing manganese and harvested after 4 h of growth and the three element co-location view respectively. The results suggested that there was a low level of manganese accumulation (Table 5.2), similar to that observed for *L. paracasei* grown in a manganese-deficient medium (Table 5.3). A lower level of phosphorus was also observed during the early log phase, suggesting that the cell was not properly developed. Similar findings were reported by Cohen et al. (2006), from their analysis of

L. plantarum WCFS1; they reported from their proteomic analysis that strengthening of the cell membrane appeared to occur during the late log to early stationary phase, based on the greater abundance of enzymes involved in fatty acid biosynthesis that are needed for the formation of phospholipids. Their finding was consistent with the present XFM results, which also suggested less phosphorus accumulation in early log phase cells and similarly low levels of phosphorus in *L. paracasei* cells grown in a manganese-deficient medium. The present results indicated that the level of manganese accumulation may have an impact on the viability, by strengthening the cell membrane. From the images (Figures 5-7–5-12), it was clear that manganese was distributed throughout the cells rather than in a deposit form, as reported previously by Archibald and Duong (1984) using SEM.

The osmolyte K^+ is important in bacterial cells as it helps to maintain turgor pressure within the cells (Burg and Ferraris, 2008). Therefore, the potassium channel was useful for outlining the bacterial cell structure. Consequently, the cell outline in the K channel served as a cell marker for all other channels. Moreover, there was a greater accumulation of potassium ions in *L. paracasei* cells after 18 h of growth in MRS medium without manganese (Table 5-3). Similarly, *L. paracasei* cells showed greater accumulation of potassium ions during the early log phase (4 h) of growth (Table 5-2). This finding is in line with previous reports, in which researchers observed increased potassium accumulation under osmotic stress conditions (Csonka, 1989). In the early log phase, bacteria are added to a new medium and attempt to accumulate potassium ions. As bacterial growth in a medium without manganese causes similar effects, there may be a relationship between manganese uptake and potassium accumulation in the cells. Table 5-3 clearly shows that the bacteria, when grown without the presence of

manganese in the medium, had an increased uptake of potassium as observed by Csonka (1989).

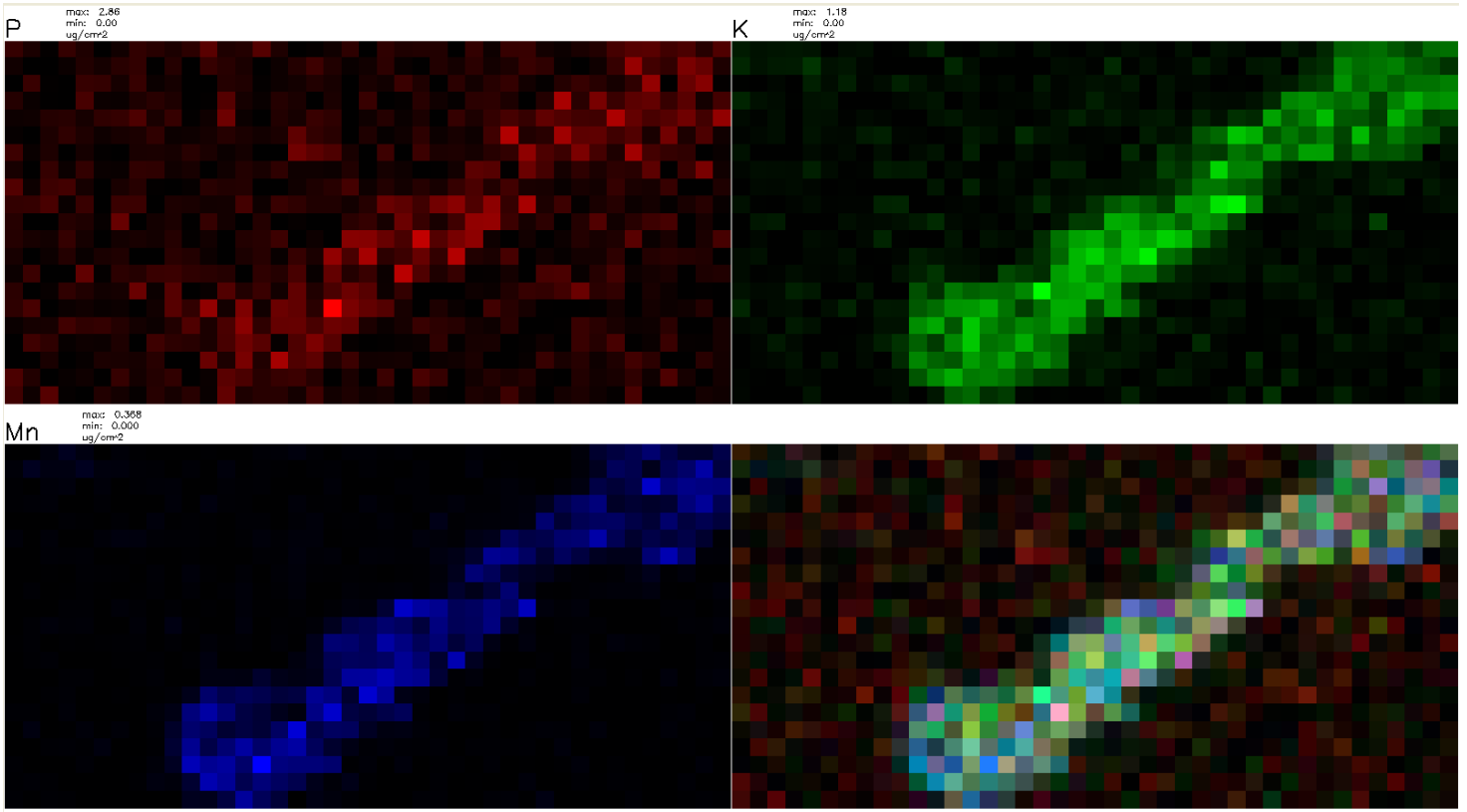

Figure 5-8 Synchrotron radiation XFM microprobe mapping of three element co-location view (phosphorus, potassium and manganese in individual channels) in a single *L. paracasei* cell grown in a manganese-rich growth medium (18 h, MRS medium). Beam spatial resolution (V × H): 0.1 μm × 0.1 μm; colour scale in counts per pixel. The image in the bottom right channel is the overlay.

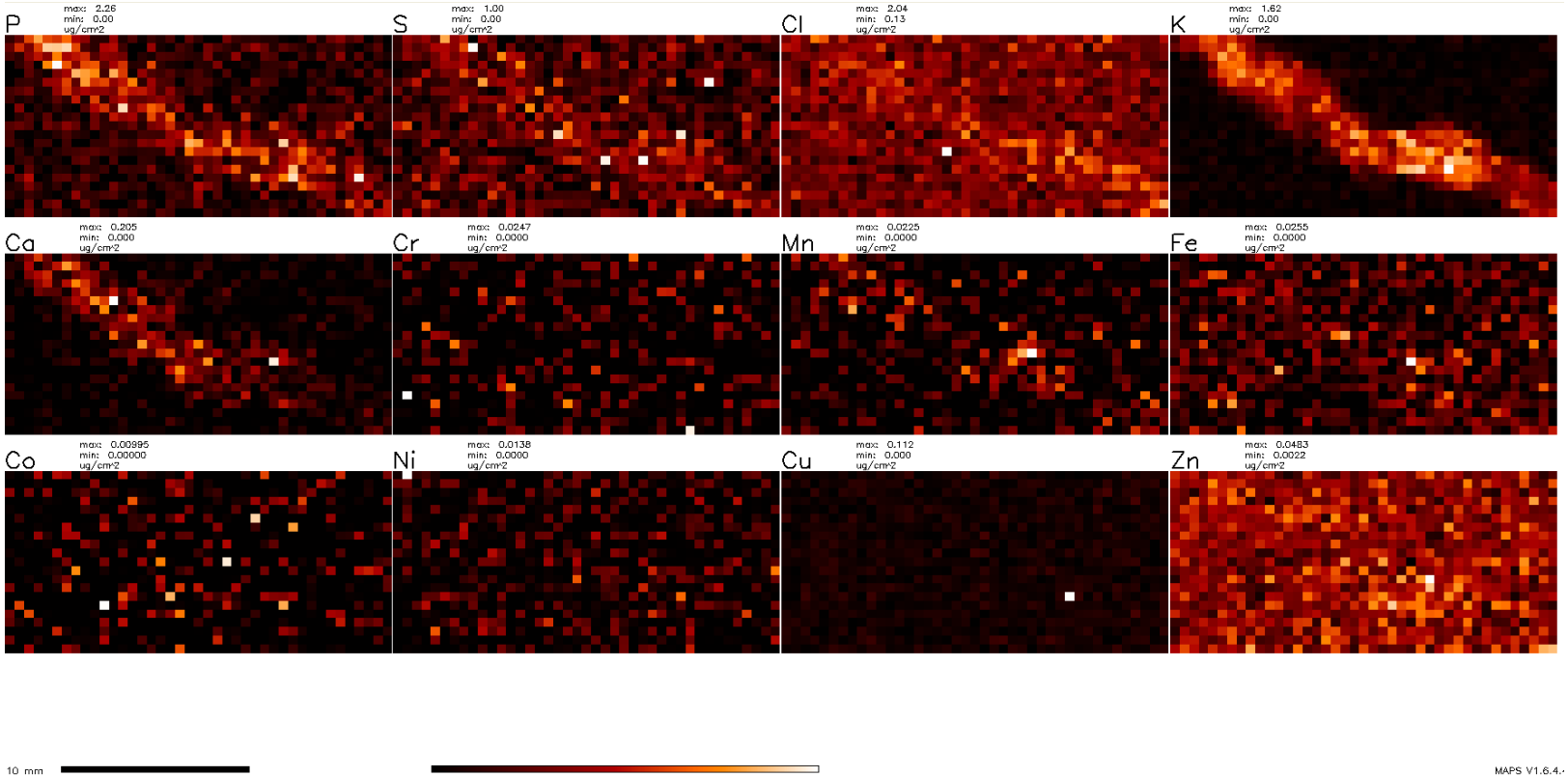

Figure 5-9 Synchrotron radiation XFM microprobe mapping of elements (phosphorus to zinc in individual channels) in a single *L. paracasei* cell grown in a manganese-deficient growth medium (18 h, MRS medium, without added manganese). Beam spatial resolution (V × H): 0.1 μm × 0.1 μm; colour scale in counts per pixel.

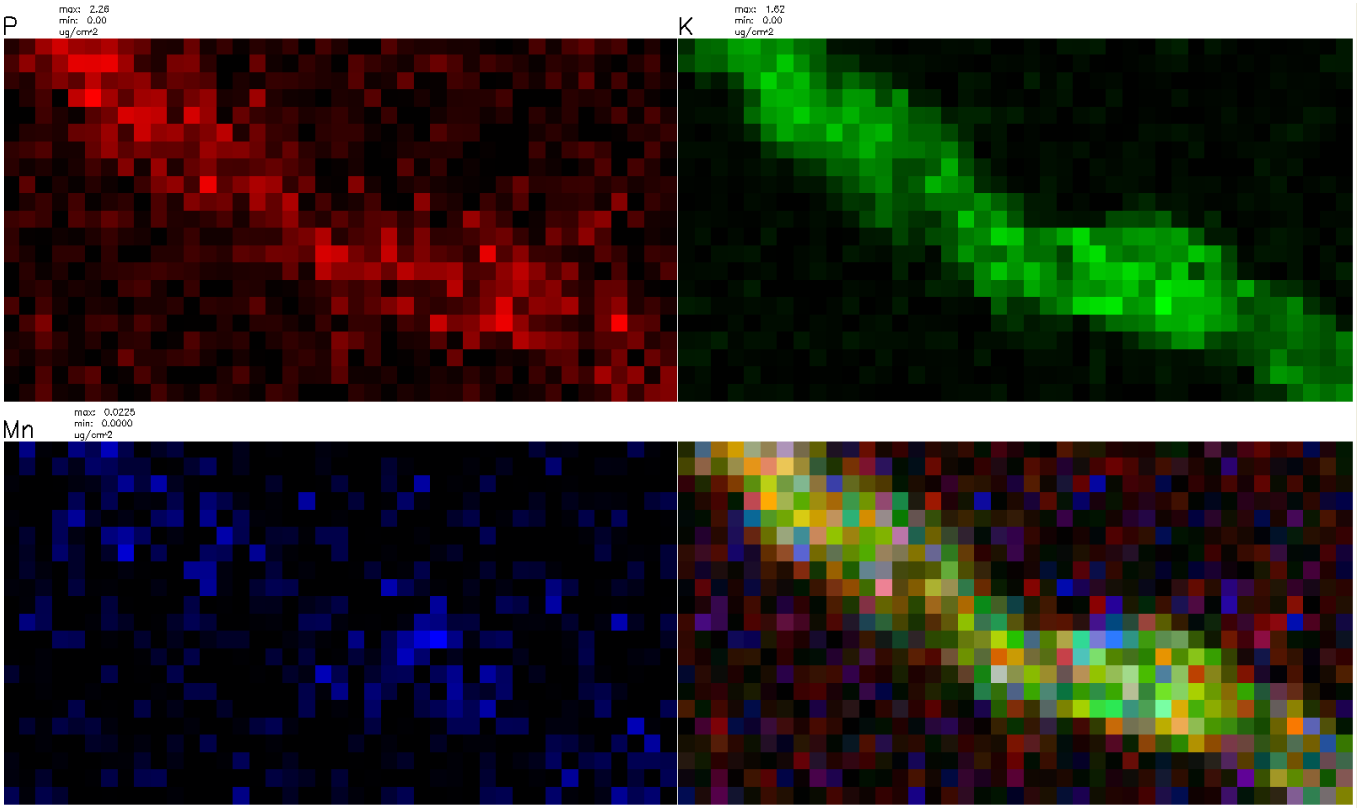

Figure 5-10 Synchrotron radiation XFM microprobe mapping of three element co-location view (phosphorus, potassium and manganese in individual channels) in a single *L. paracasei* cell grown in a manganese-deficient MRS medium (18 h, MRS medium, without added manganese). Beam spatial resolution (V × H): 0.1 µm × 0.1 µm; colour scale in counts per pixel. The image in the bottom right channel is the overlay.

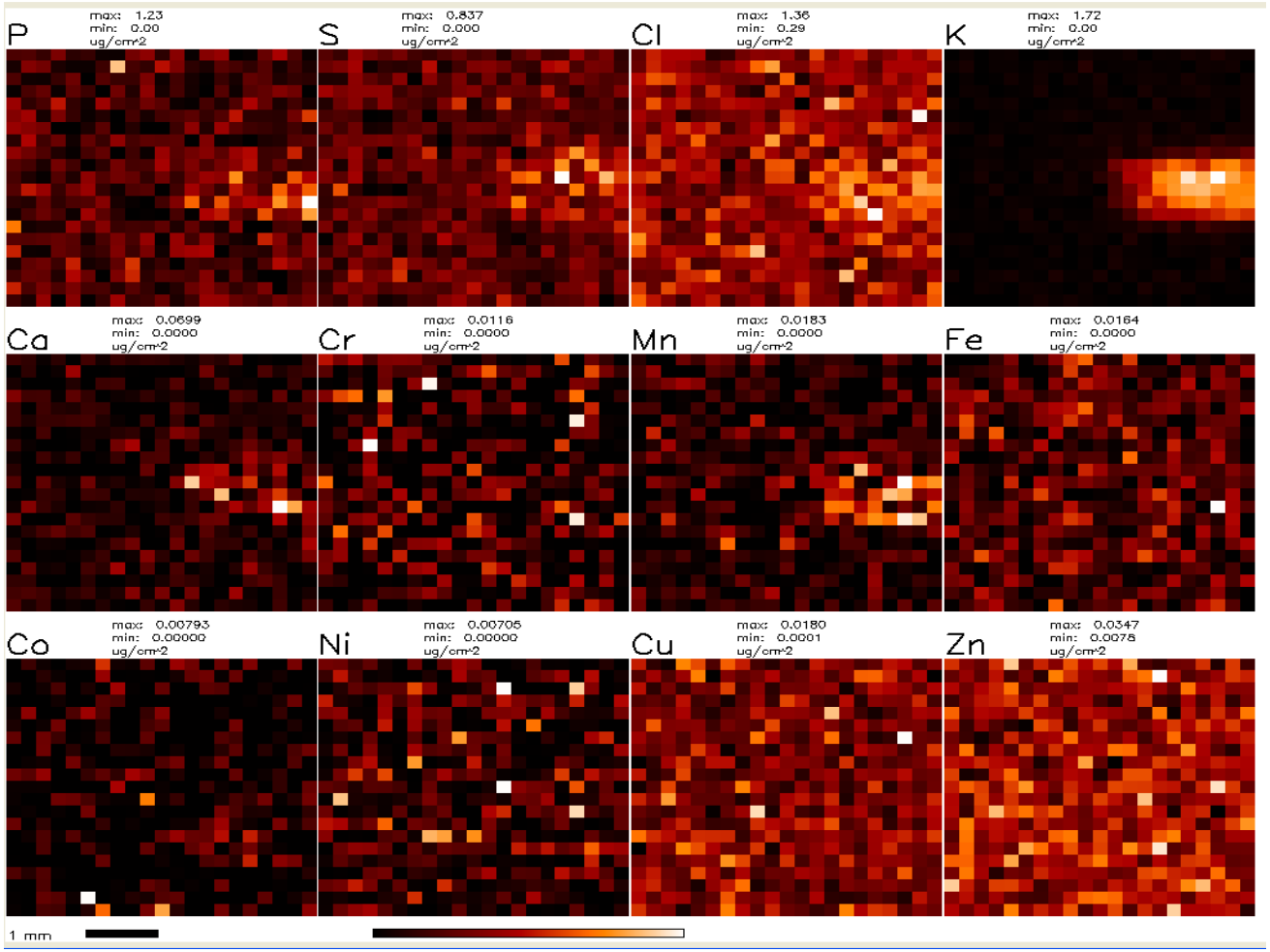

Figure 5-11 Synchrotron radiation XFM microprobe mapping of elements (phosphorus to zinc in individual channels) in a single *L. paracasei* cell grown in a manganese-rich growth medium (4 h, manganese-rich MRS medium). Beam spatial resolution (V × H): 0.1 µm × 0.1 µm; colour scale in counts per pixel.

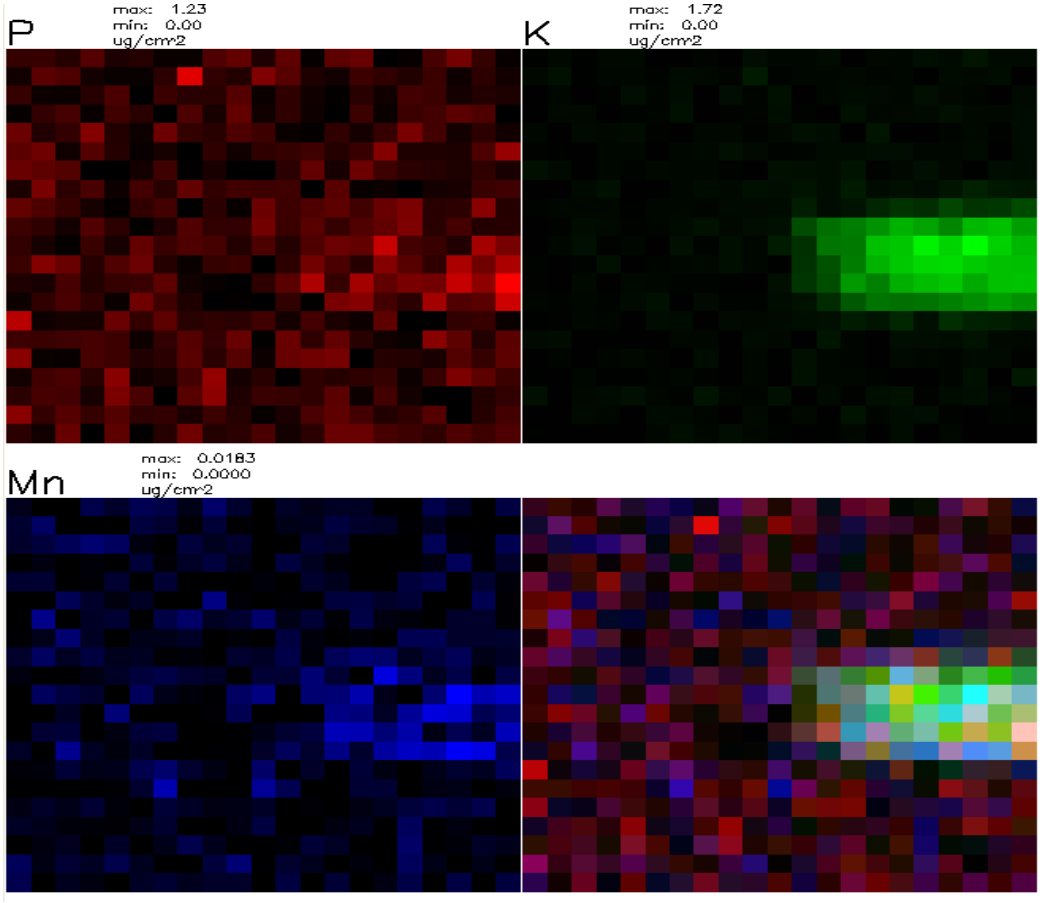

Figure 5-12 Synchrotron radiation XFM microprobe mapping of three element co-location view (phosphorus, potassium and manganese in individual channels) in a single *L. paracasei* cell grown in a manganese-rich growth medium (4 h, MRS medium). Beam spatial resolution (V × H): 0.1 μm × 0.1 μm; colour scale in counts per pixel. The image in the bottom right channel is the overlay.

Figures 5-7–5-12 suggested that there was a considerable difference in the elemental compositions of *L. paracasei* grown in an MRS medium containing manganese and harvested after 4 and 18 h and *L. paracasei* grown in an MRS medium without added manganese and also harvested after 4 h. Therefore, it was of interest to immobilize *L. paracasei* in the matrix using fluidized-bed drying and to study the bacterial viability during storage. *L. paracasei* cells harvested after 4 or 18 h of growth in MRS medium and after 18 h of growth in MRS medium that was deficient in manganese were embedded into WMP using a fluidized-bed drying technique, as outlined in Section 4.3.2.3. The aim was to understand whether there was a relationship between the viability and the accumulation of manganese in the cells.

L. paracasei, when grown in MRS medium (containing manganese) and in MRS medium (without added manganese), reached cell concentrations of 9.26 ± 0.21 and 8.70 ± 0.19 log CFU/ml respectively after 18 h of incubation. Manganese was observed in cells harvested after 4 h only when grown in an MRS medium containing manganese, at a level of 7.13 ± 0.08 log CFU/ml. The *L. paracasei* cells that were grown in MRS medium containing manganese and harvested after 18 h of growth retained viability during storage at a water activity of 0.33 and 25ºC for 15 days. However, the bacteria harvested during the early log phase and the bacteria grown in a manganese-deficient medium lost viability, with *L. paracasei* concentrations of < 4.0 log CFU/g after 15 days of storage at a water activity of 0.33 and 25ºC, as shown in Table 5-4.

Table 5-4 Viability of *L. paracasei* grown in MRS medium, with or without added manganese, and embedded in a WMP matrix, before and after fluidized-bed drying, and after storage for 15 days at a water activity (a_w) of 0.33 and 25ºC

Samples	Incubation time (h)	Harvested cells (log CFU/g)	Before drying (log CFU/g)	After drying (log CFU/g)	Storage for 15 days at 25ºC, 0.33 a_w (log CFU/g)
L. paracasei (MRS with manganese)	18	9.26 ± 0.21	9.43 ± 0.23	9.22 ± 0.08	9.19 ± 0.12
L. paracasei (MRS without manganese)	18	8.70 ± 0.19	8.84 ± 0.13	6.23 ± 0.04	< 4
L. paracasei (MRS with manganese)	4	7.13 ± 0.08	7.82 ± 0.15	5.25 ± 0.32	< 4

These results indicated that, after 18 h, the number of *L. paracasei* cells grown in the MRS medium without manganese was lower than that in the MRS medium containing manganese. Moreover, rapid bacterial death was observed in the bacteria grown in the manganese-deficient medium during the drying process and storage. There was no survival of the bacteria grown in the manganese-deficient medium after 15 days of storage. This result was consistent with previous findings, in which early log phase cells were found to be unsuitable for stabilization (Corcoran et al. 2004). In a separate experiment, additional manganese was added to the growth medium (MRS) to ascertain whether the viability of the bacteria could be improved. However, additional manganese did not have an effect on the viability of the bacteria during drying and storage. This finding was also consistent with Archibald and Duong (1984), who established that, beyond a certain level, the addition of manganese did not affect the accumulation of manganese in the cells.

These findings are also useful in the context of the regulatory status of probiotics, which has not been well established at the international level (Caselli et al. 2013). With reference to these results, it may be of interest to review the FAO guidelines for probiotic bacteria to be tolerant to gastric juice and bile salts when using MRS (or any manganese-rich medium) as the medium for growth for probiotic screening purposes during *in vitro* screening. The use of manganese in the growth medium leads to more robust bacteria compared with bacteria grown in milk, which is deficient in manganese. Therefore, the bile salt tolerance result might vary depending on whether the bacteria are grown in a manganese-rich medium or in a manganese-deficient medium. In light of the present study, it is also tempting to assume that the consumption of a food that is rich in manganese will aid in better colonization of probiotics in the gastrointestinal tract.

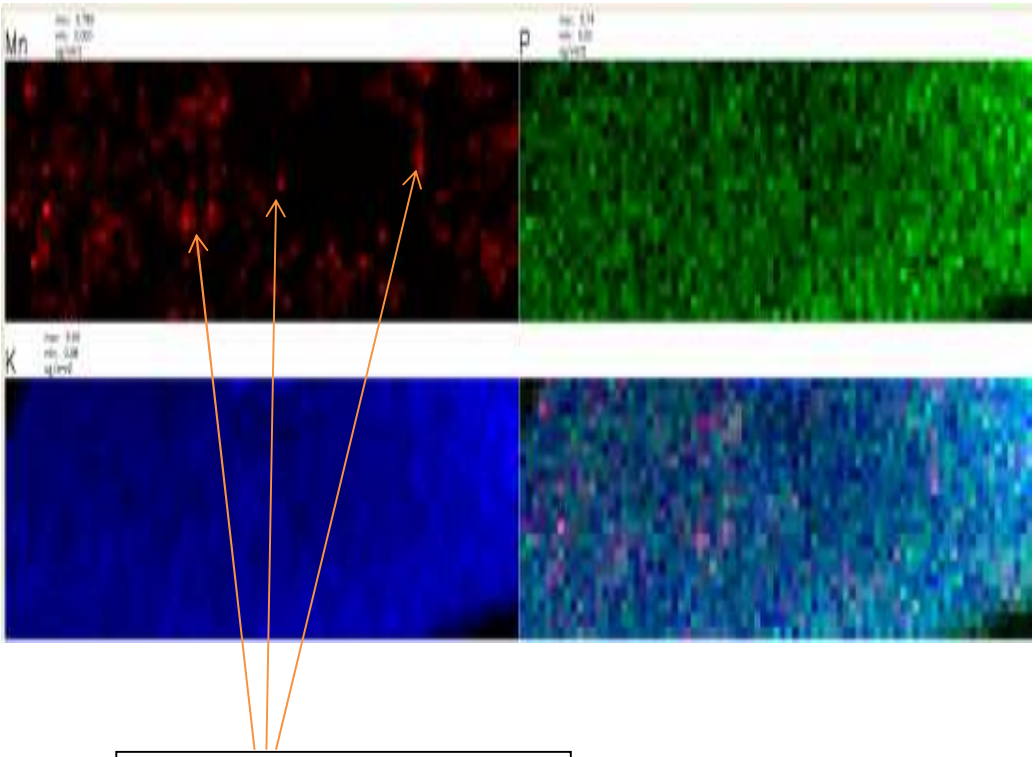

Figure 5-13 XFM mapping of freeze-dried WMP matrix containing *L. paracasei*, manganese channel with red dots representing bacteria embedded in the matrix. The phosphorus and potassium channels could not distinguish the dairy matrix from the bacteria. The WMP embedding matrix was rich in both phosphorus and potassium.

The stabilization of probiotics generally involves embedding them in a suitable matrix. The visualization of bacteria within the matrix is usually carried out using CLSM, which requires specific dyes and solvents (refer to Section 4.3.6.3). A milk-based matrix poses a challenge for a study of the location of bacteria. Synchrotron-based XFM is a novel technique for mapping the bacteria in an embedding matrix. XFM was used to study the *in*

situ location of bacteria using manganese as a tracer (Figure 5-13). With the use of XFM, the ICP–MS result (Table 5.1) was further reinforced and it was observed that no manganese signals could be obtained from the dairy matrix. Therefore, the XFM technique could be used to study the location of manganese-accumulating bacteria in the powders. Dairy matrix being rich in phosphorous and potassium saturates the XFM channels. However, if the embedding matrix is deficient in phosphorus and potassium it may be possible to acquire important information from those channels.

5.4 Conclusions

XFM, which was employed to determine changes in the elemental composition of *L. paracasei* ATCC 55544 during growth in a manganese-rich MRS medium and in an MRS medium without added manganese and as a function of the physiological growth state (early log phase versus stationary phase), revealed less manganese accumulation during the early log phase of bacterial growth than for the stationary phase cells. The lower level of manganese accumulation was related to a loss of bacterial viability during storage. These results suggest that bacteria grown in the early log phase and upon growth in a manganese-deficient medium may provide less protection to the bacteria against oxidative stress and may be unsuitable for stabilization. The present study also stresses the need to study the changes within the bacteria during storage. As manganese is important for bacterial viability during storage, any change in manganese during storage might explain any loss in bacterial viability during storage.

Chapter 6 Changes in the accumulated manganese in *Lactobacillus paracasei* embedded in milk powder matrix during storage

6.1 Introduction

Chapter 5 showed that lower levels of manganese accumulation probably lead to loss of bacterial viability during storage. The accumulation of manganese in the bacteria was shown to provide protection during drying and storage processes and this improved viability may be due to the free-radical-scavenging activity of manganese. It is a challenge to monitor the changes in bacteria during storage when they are embedded in a complex matrix comprising fat, protein and carbohydrate. However, in Chapter 5, it was established that the embedding matrix is devoid of manganese, which provides a means for tracking the changes occurring in manganese during storage. The radiation-resistant bacterium *Deinococcus radiodurans* has high cellular levels of manganese, which have been attributed to the remarkable survivability of this microorganism (Daly et al. 2004). Manganese has also been found to play an anti-oxidative role in the nematode *Caenorhabditis elegans* (Lin et al. 2006). Manganese and other elements have been found to be strictly maintained and enriched within specific cell types of *C. elegans* (McColl et al. 2012). However, there is still ambiguity over the form and the state of manganese in bacterial cells (Archibald and Fridovich, 1982b; Barnese et al. 2012).

Lactobacillus plantarum, which is similar to *Lactobacillus paracasei*, has also been found to accumulate large amounts of inorganic manganese (30–35 mM) during growth in a manganese-rich medium. This is thought to provide a defense against oxidative damage (Archibald and Duong, 1984; Watanabe et al. 2012). Barnese et al. (2012) have provided

evidence on the mechanism by which manganese inhibits superoxide in the cellular system. They have shown that, under simulated physiological conditions, both manganese(II) phosphate and manganese(II) carbonates are able to catalytically remove superoxide from the solution.

Manganese accumulation was achieved in probiotic *L. paracasei* ATCC 55544 (Chapter 5). This *L. paracasei* was then incorporated into a dairy matrix, which has been shown to stabilize the probiotic bacteria provided the water activity is kept sufficiently low (Chapter 4). However, there is interest in the state and the form of manganese during storage, which may provide a clue to further improving the viability during storage. Moreover, it is important to explore the relationship between changes in the manganese oxidation state and viability during storage. It has been observed that radiation-resistant species of *D. radiodurans* produce large amounts of manganese metallopeptide in stationary phase cells, in addition to manganese superoxide dismutase (Tabares and Un, 2013). As manganese accumulation increases during the growth stage (Chapter 5), it is of interest to study the *in vivo* composition and the protein/peptide–manganese association.

The *in vivo* relevance of manganese deposits is not understood because little is known about the cellular manganese(II) speciation. *Ex vivo* experiments by Daly et al. (2004) proposed that the radio-resistance of *D. radiodurans* is mediated by manganese(II) inorganic phosphate metabolite complexes that specifically protect cytosolic proteins against ionizing radiation. Although *ex vivo* approaches can identify potential complexes, they invariably lead to changes in intracellular equilibrium concentrations, affecting the manganese speciation (Tabares et al. 2013). This general problem that is associated with metal speciation studies may be avoided by electron spin resonance (ESR) spectroscopic

analysis of intact bacteria. Therefore, embedded bacteria (Chapter 4) that were stored under various water activity conditions were further analysed using ESR spectroscopy. However, as the ESR spectroscopy used in the current study had the limitation of not being able to detect the manganese(III) species, synchrotron-based X-ray absorption spectroscopy (XAS) was used for further investigations and to simultaneously correlate with the results obtained from ESR spectroscopy. XAS is a useful tool for studying the oxidation state of elements *in situ*, particularly by comparison with standard compounds.

In the work reported in this chapter, XAS was used to investigate the speciation in manganese accumulated in *L. paracasei* ATCC 55544 that was incorporated into a dairy matrix. The bacterial preparations were subjected to different humidity environments and storage times to vary the oxidative stress experienced. A correlation between the loss of viability of the bacteria upon storage and changes in the manganese speciation was explored.

6.2 Materials and methods

6.2.1 Bacterial growth conditions

Freshly grown cultures of *L. paracasei* and *L. plantarum* in MRS broth were harvested after 18 h. The harvested bacteria were freeze dried for an inductively coupled plasma–mass spectrometry (ICP–MS) study. They were also harvested at various stages of growth (6, 12, 18 and 24 h) and the change in the manganese speciation was monitored.

To simulate bacterial death, the harvested cells were either sonicated or treated with ethanol (70%) and then further characterized by XAS. *L. paracasei* cells were lysed by sonication

(MSE Soniprep 150 sonicator, MSE, UK) by applying 20 s pulses at a power output of 14 µm for 20 cycles. The viability of the microorganisms was tested as described previously.

6.2.2 Characterization of manganese in the bacteria

Bacteria embedded and stored under different water activity conditions (from Chapter 4) were studied for any changes observed in the manganese within the bacteria.

6.2.3 Electron spin resonance spectroscopy

Electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR), is a spectroscopic technique that has the ability to detect species that have unpaired electrons. ESR can detect both free radical species and paramagnetic species at the same time. ESR measures the transition between the electron spin energy levels, in a magnetic field, with the transition being induced by microwave frequency radiation. The required frequency of radiation is dependent upon the strength of the magnetic field. The common field strengths used are 0.34 and 1.24 T and 9.5 and 35 GHz in the microwave region.

Like a proton, an electron has a spin, which gives it a magnetic property known as a magnetic moment. Under the influence of an external magnetic field, the paramagnetic electrons can orient either parallel or antiparallel to the direction of the magnetic field. This result in the formation of two distinct energy levels for the unpaired electrons and measurements are made as the electrons are driven between the two levels.

A Varian E-104A EPR spectrometer equipped with an E-257 variable-temperature controller and operating at about 9.0 GHz was used in the present study. Small samples of bacterial powder were placed in EPR tubes (Wilmad Labglass, USA) and analysed. The

typical operating parameters were as follows: microwave frequency 8.99 GHz; microwave power 30 mW; modulation frequency 100 kHz; sweep time 2 min. The sweep width was 2000 G, and the value of the centre of the field was 3000 G. The spectral g values were calibrated with (diphenylpicryl) hydrazyl (DPPH) as a standard. The spectral data acquired were digitalized using a plot digitalizer, after image calibration (University of South Albama, n.d.).

6.2.4 Transmission electron microscopy–electron energy loss spectroscopy and energy-dispersive X-ray spectroscopy

Manganese is an electron-opaque metal and its uptake into bacterial cells is likely to show up under an electron microscope as an electron-dense region. Bacterial powder samples were fixed with 2% (w/v) formaldehyde and 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. After three washes in the same buffer, the samples were post-fixed with 1% (w/v) OsO_4 in the same buffer for 1 h at room temperature. The three buffer washes were repeated. The samples were then dehydrated using an water/acetone series (25, 50, 75, 95 and 100%) and kept in each gradient for 10 min and in the 100% acetone for 2 h. The samples were first embedded in an acetone/resin (Procure 812) mixture (50%) on a stirrer overnight, and then the acetone/resin mixture was replaced with fresh 100% resin for another 8 h on the stirrer. The samples were finally mounted in 100% fresh resin at 60ºC for 48 h.

Sections (1 μm in thickness) were cut from the trimmed resin blocks using a glass knife and an Ultramicrotome (Leica, Austria). They were heat mounted on to glass slides, stained with 0.05% toluidine blue and viewed under a light microscope (Olympus BX51, Japan).

Digital images of the sections were taken and areas of interest were chosen for examination by transmission electron microscopy (TEM).

Ultra-thin sections (100 nm) were cut using a diamond knife and an Ultramicrotome. They were collected on a copper grid. The sections were stained with saturated uranyl acetate in 50% ethanol for 4 min.

TEM elemental analysis for electron-dense manganese particles in bacteria was carried out on two transmission electron microscopes both working at 120 kV, which is suitable for biological materials and prevents samples from being damaged by electron radiation. Conventional energy-dispersive X-ray spectroscopy (EDS) spectra for the elements manganese and sulphur were collected using a JEOL-1400 equipped Oxford EDS detector, which was working in a liquid nitrogen environment (lower than 173 K). Elemental mapping for manganese was carried out on a small square area with image drift auto-correction. Conventional electron energy loss spectroscopy (EELS) spectra were obtained with a CM-120 equipped with a Gatan GIF detector.

EELS was used to determine the oxidation state of the manganese in bacteria, using a chemical shift of L 2,3 edge and the ratio of L3 to L2.

6.2.5 X-ray absorption spectroscopy at Australian Synchrotron facility

6.2.5.1 X-ray absorption spectroscopy

X-ray absorption spectroscopy (XAS) techniques collect information about energy emissions and electron excitations initiated by the absorption of X-ray energy, yielding information on bond lengths, coordination numbers, the local coordination geometry and

the oxidation state of atoms for a wide range of solid and liquid systems. XAS experiments require an intense, tunable photon source that is available only at synchrotron facilities. Principle: X-rays are ionizing radiation and possess the ability to remove the core electron from an atom. Each core shell has a distinct binding energy and, when the X-ray is scanned through the binding energy of a core shell, there is a rapid increase in the absorption cross section. This results in an absorption edge, where each edge represents a different core electron binding energy. The edges are referred to according to the principal quantum number of the electron that is excited: K (n = 1); L (n = 2); M (n = 3). The core electron binding energy increases with the atomic number; the K edge for manganese is 6.5390 keV.

The structure near the edge is known as the X-ray absorption near-edge structure (XANES). An oscillation above the edge, 1000 eV or more, is known as an extended X-ray absorption fine structure (EXAFS). A XANES region provides information on the oxidation state and the geometry, whereas an EXAFS region provides information on the electron density and a quantitative determination of bond lengths and the coordination geometry surrounding the atom of interest (Australian Synchrotron, 2012).

6.2.5.2 Data analysis

Raw data from samples studied in the XAS beamline were collected, saved in binary ‘mda’ format and converted to ascii format for analysis.

Data analysis was carried using the IFEFFIT package (Athena).

6.2.5.2.1 Technical information

Photon delivery system

Source	1.9T Wiggler
Available Energy range	5 - 35 keV
Optimal Energy range	Mode 1: 5 - 9 keV using Si(111) Mode 2: 8.5 - 18.5 keV using Si(111) Mode 3: 15 - 35 keV using Si(311
Resolution *delta*E/E	Crystal dependent: ~ $1.5x10^{-4}$ using Si(111) ~ $0.4x10^{-4}$ using Si(311)
Nominal beam size at sample	~ 0.25 by 0.25 mm fully focussed
Photon flux at sample	10^{10} to 10^{12} ph/s using Si(111) 10^{9} to 10^{11} ph/s using Si(311)
Harmonic content at 5-18 keV	< 10^{-5}

6.2.5.2.2 Sample handling and detectors (XAS)

The experimental hutch (Hutch B at Australian Synchrotron) was used for the fluorescence XAS of ‘standard samples’ in a standard plastic sample holder (aluminium sample holders were not used in the present study to minimize noise). A 100 element solid state

germanium fluorescence detector was used in the present study to minimize the beam degradation of the sample.

Detector Hutch B – highlights
Optical table 2.4 m by 1.2 m
100 element germanium fluorescence detector
Three ion chambers
Cryostat and room temperature holder
Harmonic rejection mirror
Fast shutter

Embedded bacterial powders that were produced by fluidized-bed drying, spray drying and freeze drying (Chapter 4) were further studied using XAS. Powder samples stored under controlled water activity conditions were studied to evaluate the influence of oxidative stress conditions on the loss of viability during storage and changes in the manganese oxidation state. The bacteria harvested during various stages of growth were also used to study the changes in manganese. Also, an attempt was made to simulate bacterial death by means of sonication or ethanol treatment. Given the successful XANES and EXAFS study of plant manganese hyper-accumulators (Fernando et al. 2010) and the parallels to our

bacterial system, the methodology of Fernando et al. (2010) was followed. The XAS study focused not only on *in situ* manganese speciation and structure but also on the changes that occur within the manganese bodies under different levels of oxidative stress and their correlation with the loss of viability upon storage. Initially, XANES measurements were made to characterize the oxidation state and the overall ligand geometry of manganese under different water activity conditions during storage and the resultant bacterial viability. Then EXAFS studies were carried out on selected samples to characterize the nature of the manganese environment in viable and non-viable bacteria.

6.2.5.3 Synthesis of model compounds

To understand the state of the released manganese bodies upon bacterial death, XANES spectra of the model compounds manganese(II) phosphate, manganese(II) hydrogen phosphate, manganese(II) acetate, manganese(II) sulphate, manganese(II) chloride, manganese(II) carbonate, manganese(II) oxide, manganese(III) acetate and manganese(III) phosphate were studied. These model compounds were considered to aid in modelling the *in vivo* speciation of manganese. The selection of phosphate, hydrogen phosphate, carbonate, acetate, chloride and sulphate was based on previous publications and our assumptions. Manganese(II) acetate, manganese(II) sulphate, manganese(II) chloride and manganese(III) acetate were purchased from Sigma-Aldrich Chemical Co. (USA).

Manganese(II) phosphate was made by the addition of a solution of 0.16 g (1 mmol) of Na_2HPO_4 in 8 ml of water to a solution of 0.25 g (1.5 mmol) of $MnSO_4.H_2O$ (i). The resulting precipitate was separated by filtration, washed with water and then air dried.

$$2Na_2HPO_4.H_2O + 3MnSO_4.H_2O \rightarrow Mn_3(PO_4)_2 + H_2SO_4 + 2Na_2SO_4 + 2H_2O \qquad \text{(i)}$$

Manganese(II) hydrogen phosphate was prepared by the addition of 0.66 ml (1.15 g, 10 mmol) of 85% phosphoric acid to a stirred solution of 1.23 g (5 mmol) of manganese(II) acetate tetrahydrate in 20 ml of water in a 50 ml round bottom flask (ii). The flask was warmed in a 50°C oil bath with stirring until all the manganese(II) acetate tetrahydrate had dissolved to give a clear slightly pink solution. The stirrer bar was removed and the flask was set aside; large gem-like crystals had grown after 48 h. The crystals were removed and air dried on a filter paper, to give 0.134 g of pale pink crystals.

$$Mn(CH_3COO)_2.4H_2O + 2H_3PO_4 \rightarrow Mn(HPO_4)^{2-}.(H_2O)_3 + 2\,CH_3COOH + H_2O \quad \text{....(ii)}$$

Manganese(II) carbonate was prepared by the addition of a solution of 2.1 g (19.8 mmol) of sodium carbonate in 25 ml of water to a solution of $Mn(NO_3)_2.6H_2O$ (5.7 g, 19.8 mmol) in 10 ml of water and mixed well (iii). A colourless precipitate was formed, which was filtered, washed well with water, then ethanol and then ether and air dried to give 1.34 g of a white powder.

$$Mn(NO_3)_2.6H_2O + Na_2CO_3 \rightarrow MnCO_3 + 2NaNO_3 + 6H_2O \quad \text{....(iii)}$$

Manganese(III) phosphate was prepared by the addition of 5 ml of 85% H_3PO_4 to 5.7 g of $Mn(NO_3)_2.6H_2O$ in 4.2 ml of water in a 50 ml round bottom flask, followed by the addition of 20 ml of ethanol. The reaction mixture was placed in an oil bath and stirred at 500 rev/min and 40ºC for 2 h (iv). The gray/green precipitate was filtered on a glass sinter, washed well with water and then air dried overnight to give 0.66 g of a gray/green powder.

$$3Mn(NO_3)_2 + 2H_3PO_4 \rightarrow Mn_3(PO_4)_2 + 6HNO_3 + 3MnPo_4 + 3O_2^- \quad \text{....(iv)}$$

6.2.5.4 XANES and EXAFS set-up

For the XANES studies, the manganese compounds were run as solid samples, dispersed in boron nitride powder, (refer Appendix Table B1) (to aid in the modelling of solid manganese deposits in the viable bacteria). All biological samples were premounted in XAS plastic sample holders of 1 mm thickness. Repeated XANES scans (2–4) were inspected for loss of signal because of radiation damage; the time to radiation damage determined the time for the collection of EXAFS data per sample. All samples were examined by XANES at the manganese K edge in the energy range 6525–6600 eV. Based on the XANES results, selected samples were analyzed further by EXAFS over the energy range 6525–7130 eV (to k ~ 12). Data were collected at ~ 20 K to minimize radiation damage and to maximize the high resolution signal.

6.3 Results and discussion

In an earlier study (Chapter 4), it was observed that bacteria stored at low relative humidity had better protection than bacteria stored at high relative humidity. The ability of *L. paracasei* to maintain viability may be enhanced by the organism accumulating manganese, which can act as a free radical scavenger. It has been observed that *Lactobacillus* sp. may accumulate large amounts of manganese, which provides protection to the bacteria against oxidative damage (Archibald and Fridovich, 1981a, 1981b; Barnese et al. 2008, 2012; Daly et al. 2004). Recently, Barnese et al. (2012) provided systematic evidence on the mechanism and demonstrated how manganese(II) combats superoxide in cellular systems. Therefore, the first step was to ascertain the presence of manganese in *L. paracasei.* Chapter 5 describes how freshly harvested bacteria (*L. paracasei*) were freeze dried and

studied using EDS and how ICP–MS confirmed the accumulation of manganese. Further, it was established using XFM that the milk powder matrix used for embedding the bacteria did not contain manganese and that manganese had an influence on the viability of the bacteria during storage. The contribution of the changes in manganese to the maintenance of the viability of the bacteria in a milk powder matrix was therefore thought to be worthy of further investigation. ESR spectroscopy was used to study the changes in manganese during storage at low and high water activity. An ICP–MS study (Section 5.3.1) had revealed that ESR-sensitive elements, such as iron and copper, which could interfere with the signal, were present in the cells in very minute amounts compared with manganese.

Manganese was present in the encapsulating matrix at a low level (0.21 mg/kg), which would not influence the ESR spectrum of the encapsulated bacteria; the amount of manganese detected in the bacteria embedded in whole milk powder (WMP), the dairy matrix, was 71 mg/kg. A high amount of phosphorus in the bacteria was shown by ICP–OES (optical emission spectroscopy) (Table 5.1), suggesting the likelihood of manganese being present as manganese phosphate, as reported previously (Archibald and Duong, 1984).

Powders stored at lower water activities 0.11 a_w and 0.33 a_w had broad ESR spectra (Figure 6-1 a, b, d, e, g, h). Freeze-dried and spray-dried powders stored at 0.52 a_w had hyperfine splitting (Figure 6-1 c, i), whereas fluidized-bed-dried powder stored at a_w 0.52 had no hyperfine splitting (Figure 6-1 f).

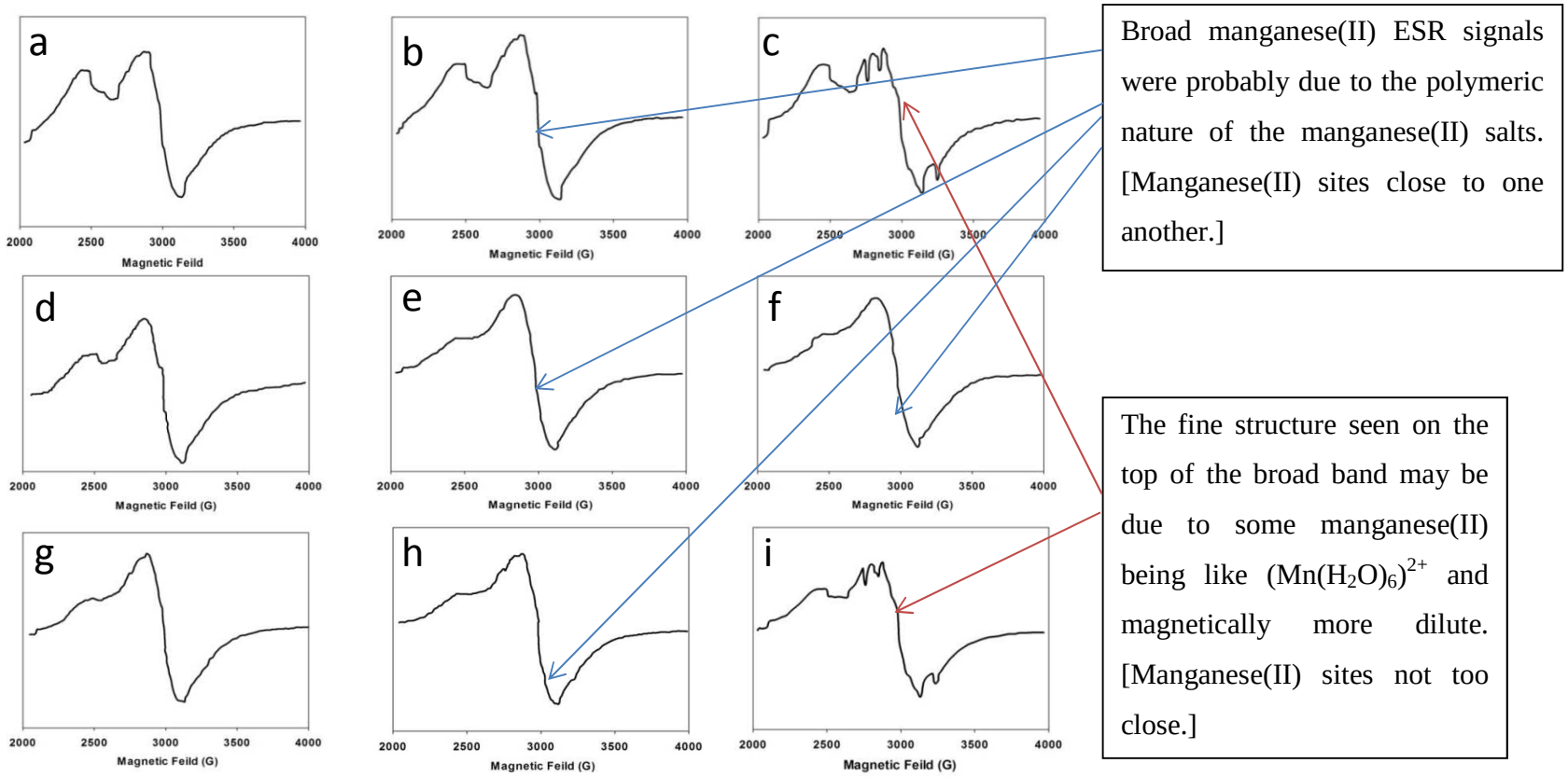

Figure 6-1 Second derivative ESR spectra of freeze-dried, fluidized-bed-dried and spray-dried bacterial powders after 1 month of storage at 25°C and controlled relative humidity conditions of 0.11 a_w (a, d, g), 0.33 a_w (b, e, h) and 0.52 a_w (c, f, i) .

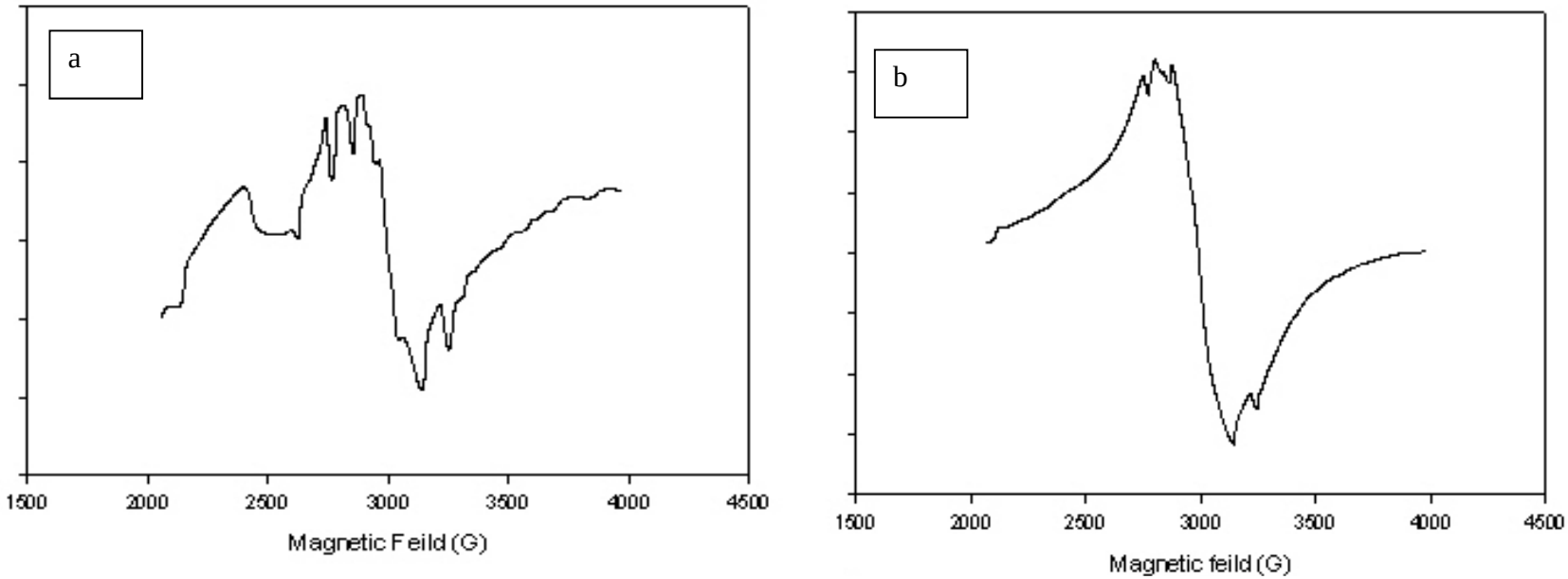

Figure 6-2 ESR spectra of (a) freshly harvested bacteria and (b) freeze-dried bacteria without the WMP dairy matrix.

The ESR spectra of the bacterial powders were obtained at room temperature. However, to acquire an ESR signal from the freshly harvested bacteria without the matrix, it was necessary to cool the sample to –160°C. The spectrum obtained (Figure 6-2a) had hyperfine splitting, similar to that observed for the spray-dried and freeze-dried powders stored at 0.52 a_w. The ESR spectrum of freeze-dried bacteria without the matrix (Figure 6-2b) also showed hyperfine splitting. The ESR spectrum of WMP showed no manganese and provided sufficient evidence that the spectrum originated from the bacteria and not from the matrix.

To validate that the spectrum originated only from manganese and not from other transition metal elements, the g value of the ESR spectrum was checked. Each transition metal has a specific g value and calculation of the g value provides a means to identify the metal. A DPPH standard was used to calculate the g value (refer Appendix Figure B3), which was found to be '2.0'. Manganese, as manganese(II), has a characteristic ESR signal with a g factor close to 2.0 and the possibility of eight-line hyperfine splitting from the I = 7/2 ^{55}Mn nucleus. Free manganese probably existed in the freeze-dried and spray-dried powders stored at a water activity of 0.52, resulting in the hyperfine splitting (water surrounding manganese was released from the dead bacteria) (Puskin et al. 1980). However, such hyperfine splitting was not observed for the fluidized-bed-dried powder, possibly because of the differences in its structure, which was consistent with the analytical results obtained from Fourier transform infrared, Raman and nuclear magnetic resonance spectroscopy, as observed in Chapter 4.

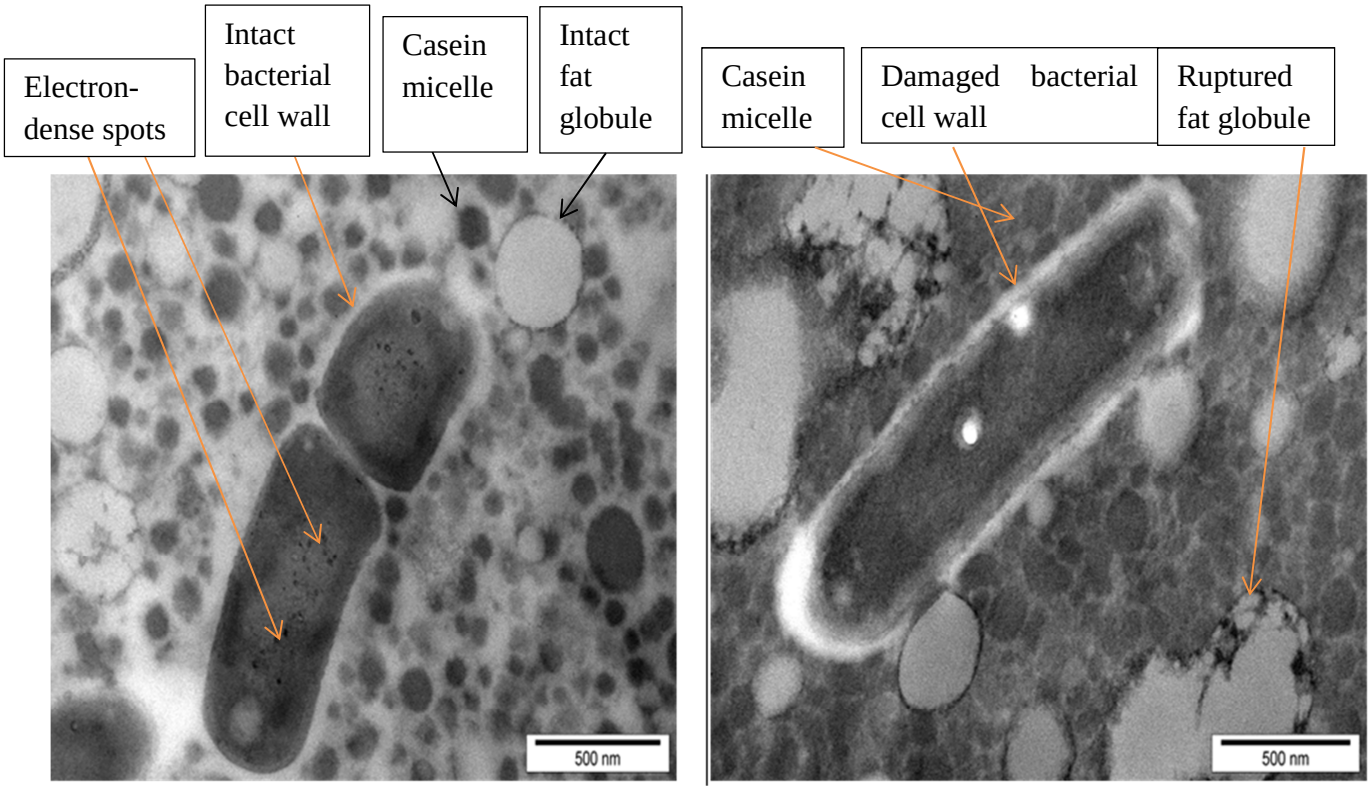

Figure 6-3 Left-hand panel: electron-dense spots observed in bacteria embedded in WMP stored at a_w 0.11. Right-hand panel: an absence of electron-dense spots for a sample stored at a_w 0.52.

TEM was used to visualize the manganese deposits and to study the differences between samples stored at 0.11 a_w and 0.52 a_w using EELS and EDS. The TEM image showed that the sample stored at 0.52 a_w had a characteristic mobility of the fat, whereas no such mobility was observed in the sample stored at 0.11 a_w (Figure 6-3). Moreover, damage to the bacterial cell membrane was evident in the sample stored at 0.52 a_w. Further, manganese deposits (electron-dense dark spots) were clearly evident in the sample stored at 0.11 a_w, whereas there was loss of the manganese in the sample stored at 0.52 a_w. A possible explanation might be that bacterial death results in a lowering of the bacterial cytosolic pH with resultant solubilization of the manganese. Archibald and Duong (1984)

shed light on the possible reason for the observed difference. Their study highlighted that formaldehyde (1% v/v) or glutaraldehyde (5% v/v) was able to seal the cells, completely preventing the loss of manganese. However, in cells treated with toluene (which disrupts the cell membrane), the addition of glutaraldehyde or formaldehyde did not prevent the exit of manganese from the cells. The observed difference in the TEM images may have been due to oxidation of the cell membrane resulting in the loss of manganese during the sample preparation step.

This cell membrane damage and the presence of manganese led to a study of manganese(III) formation during storage of the bacterial powders at higher water activity (0.52 a_w). It must be mentioned that manganese(III) has previously been reported to cause oxidative damage to neuron cells (Gunter et al. 2005).

Therefore, it became evident that determination of the oxidation state of manganese might provide a rationale for the loss of bacterial viability upon storage at higher water activity (0.52 a_w). An EELS instrument attached to a transmission electron microscope was used to determine the oxidation state. However, EELS was unable to detect the core loss peaks from manganese. The EELS spectrum, acquired in a range from 400 to 900 eV, revealed only the O-K edge (532 eV); the manganese L2 and L3 edges at 640 eV and 651 eV could not be observed (Figure 6-4).

As EELS data could not be obtained, an attempt was made to determine the presence of manganese in the TEM spots using EDS. EDS elemental maps of carbon (C), nitrogen (N), oxygen (O), sulphur (S) and manganese (Mn) in the sectioned bacteria are shown in Figure 6-5. However, EDS was unable to distinguish small manganese particles (less than 20 nm).

One possibility was that, because the bacterial samples had high electron irradiation sensitivity, they could not stand a long exposure to electron radiation, when EDS or EELS was applied, even though they were observed at under 120 keV and with the objective aperture inserted. Further, the nanosize of the particles, beyond the spatial resolution of the CM-120 EELS and JEOL-1400 EDS techniques, might have resulted in the non-detection.

The presence of manganese in the bacteria was confirmed by EDS combined with SEM, (ICP–MS) and XFM. Further, ESR spectroscopy revealed differences in the manganese spectra during storage. TEM was able to identify electron-dense manganese bodies within the bacteria, but further characterization of these bodies using EELS and EDS combined with TEM was not possible. Differences in the manganese ESR spectrum with increased bacterial death was also observed but the techniques were unable to identify the presence of manganese(III), let alone the ratio of manganese(II) to manganese(III). Manganese(III) (generally undetectable by ESR at liquid nitrogen temperature) is known to oxidize important cellular components (Gunter et al. 2006). It was therefore of interest to explore further the changes that occur within the manganese bodies under different levels of oxidative stress (comparison of samples stored at 0.11 a_w and 0.52 a_w) and to correlate these changes with the loss of viability upon storage.

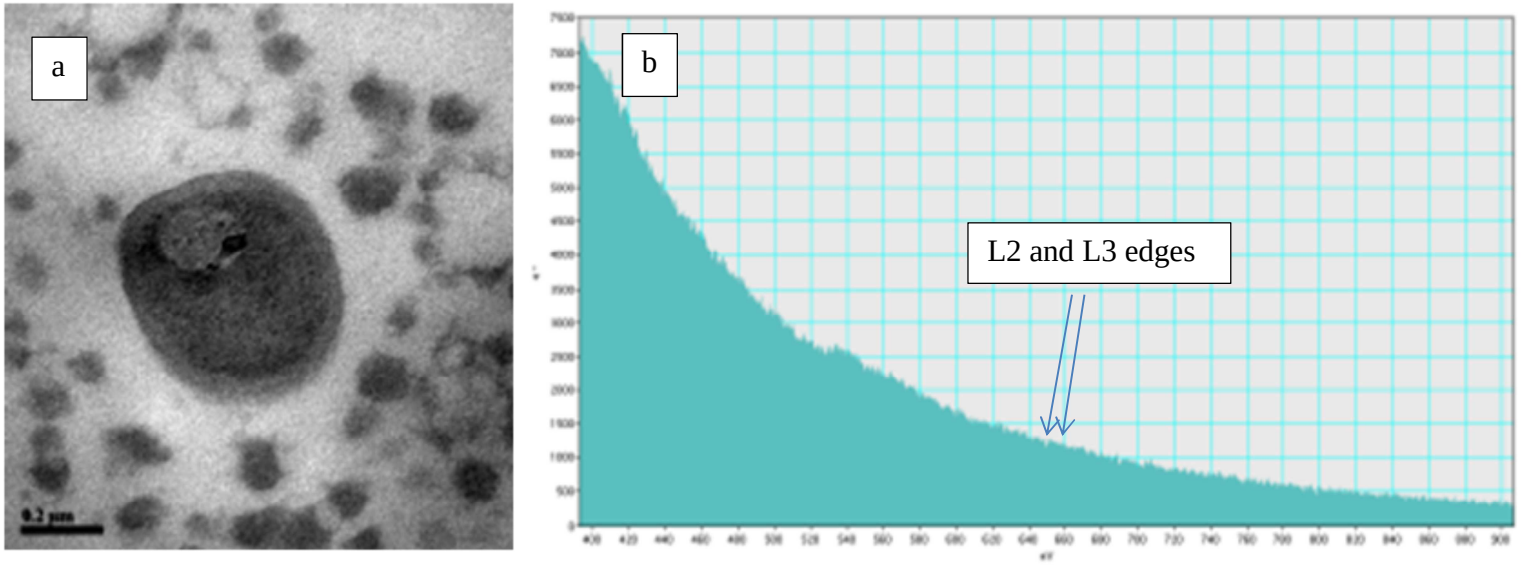

Figure 6-4 (a) TEM field image of bacterial section (electron-dense points inside the cell) and (b) the corresponding EEL spectrum in a range from 400 to 900 eV. The O-K edge (532 eV) could be observed. The manganese L2 and L3 edges at 640 eV and 651 eV could not be observed in this spectrum.

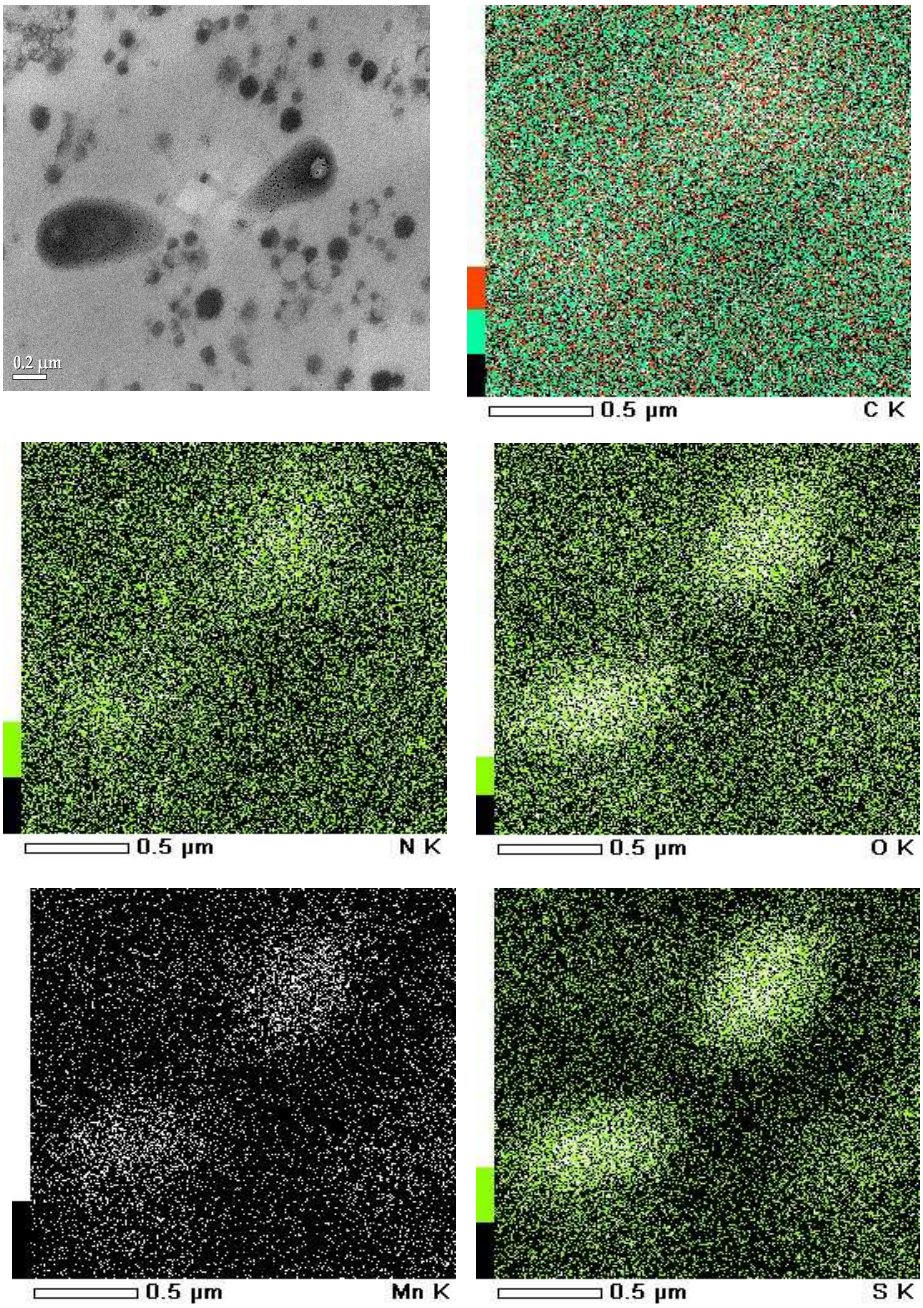

Figure 6-5 EDS elemental maps of dead bacteria showing the five elements C, N, O, S and Mn. The noise or background is apparent. The Mn and S maps provide low spatial resolution.

To study at this high sensitivity, the high signal to noise ratio of EXAFS spectroscopy, such as that provided by a synchrotron, was used. For the XAS study, the set of model compounds was carefully chosen to represent likely complexes in which manganese(II) and manganese(III) could be bound to bacterial samples. The seven standards used in the present study were manganese(II) phosphate, manganese(II) acetate, manganese(II) sulphate, manganese (II) chloride, manganese(II) carbonate, manganese(III) acetate and manganese(III) phosphate, and these were chosen to fit the spectrum of the freeze-dried *L. paracasei.*

Figure 6-6 shows the edge-normalized XANES spectra of this set of manganese(II) and manganese(III) model compounds. The XANES spectra of the manganese(II) compounds appeared to be similar to each other, but different from those of the two manganese(III) compounds, as reported previously (Fernando et al. 2010; Gunter et al. 2006)

Figure 6-7(a) compares the edge-normalized XANES spectrum of manganese in *L. paracasei* cells with the XANES spectra of a set of model compounds. The sample spectrum closely resembled that of the manganese(II) model compound but was distinctly different from that of the manganese(III) model compound. The small pre-edge peak at 6540.6 eV, the intense peak at 6553 eV and the spectral shape on the high energy side of the bacterial samples are indicative of an octahedral or pseudo-octahedral coordination environment around manganese(II) (Belli et al. 1980).

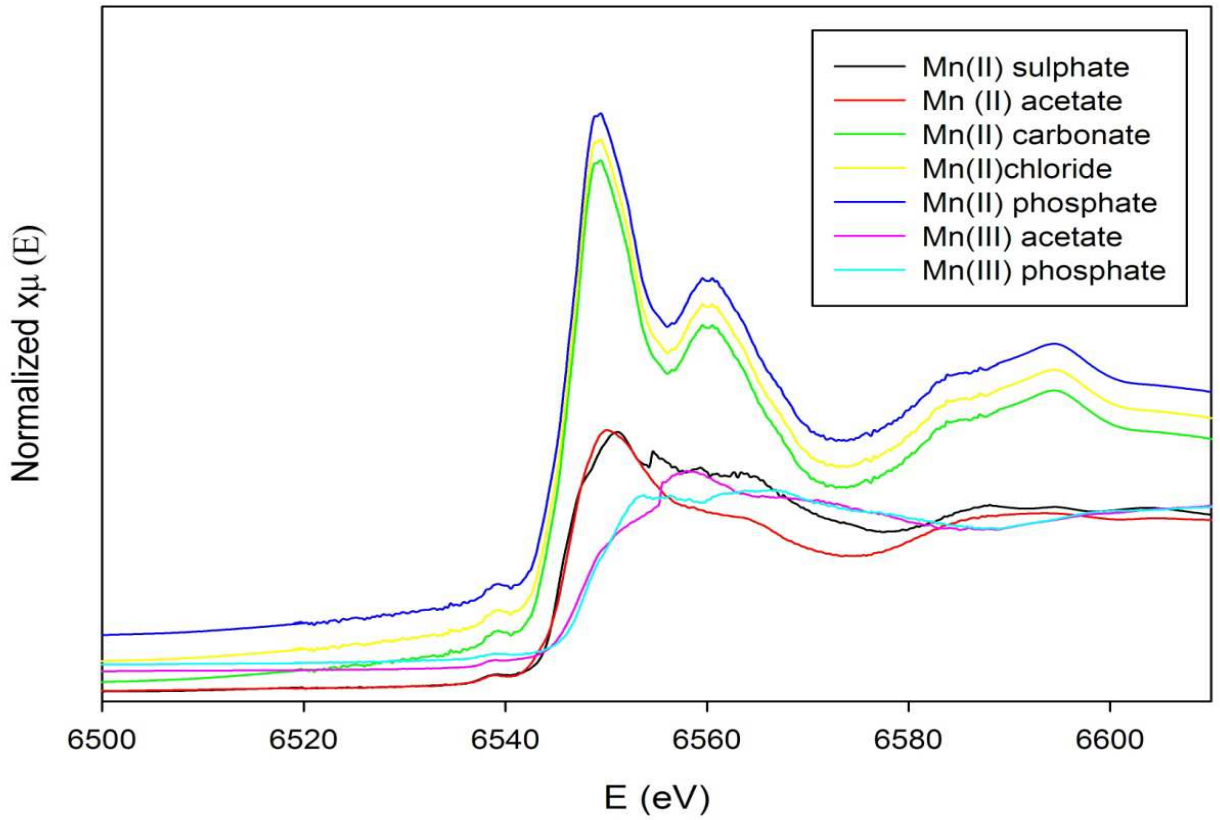

Figure 6-6 XANES absorption spectra of a set of model compounds – manganese(II) sulphate, manganese(II) acetate, manganese(II) carbonate, manganese(II) chloride, manganese(II) phosphate, manganese(III) acetate and manganese(III) phosphate.

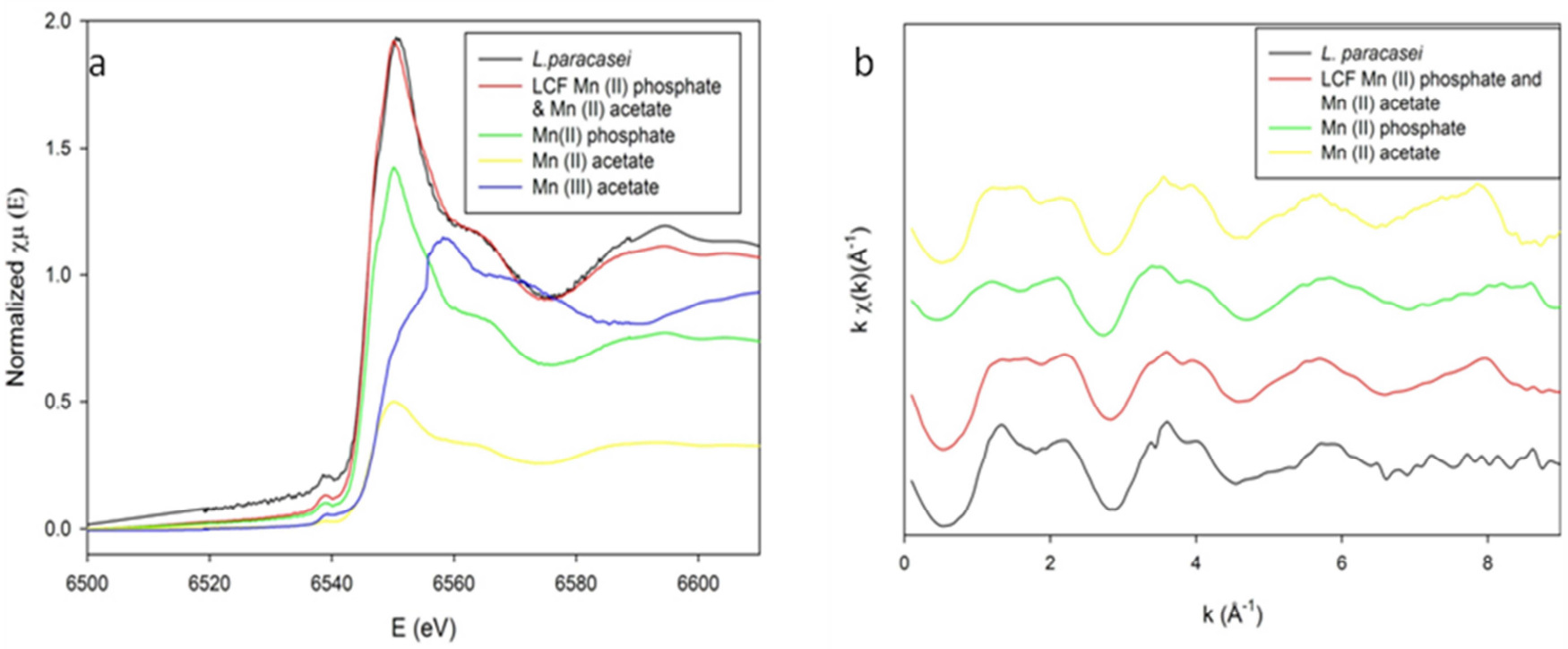

Figure 6-7 (a) XANES absorption spectra of freshly harvested *L. paracasei*: linear combination fit of manganese(II) phosphate and manganese(II) acetate, manganese(II) phosphate, manganese(II) acetate and manganese(III) acetate. (b) EXAFS spectra of *L. paracasei*: linear combination fit of manganese(II) phosphate and manganese(II) acetate, manganese(II) phosphate and manganese(II) acetate.

The bacterial XANES spectrum (*L. paracasei* incubated for 18 h in a medium containing 224 mM manganese) was compared with the spectra of the model compounds. A mixture of 0.695 manganese(II) phosphate + 0.295 manganese(II) acetate was found to best fit the manganese present in the bacteria; this fit had an R factor of less than 0.002, i.e. less than the 0.05 that is normally considered to be a good fit. The R factors for all the other compounds, including the manganese(III) model compound, did not indicate good fits. Figure 6-7(b) provides insight into the EXAFS spectra of the model compounds in relation to *L. paracasei*.

Figure 6-8(a) compares the spectra from *L. paracasei* incubated for 12 and 18 h in a growth medium (MRS) containing 224 mM of manganese. After 4 h of incubation, there was no manganese uptake, resulting in no spectrum. The results suggested that increased manganese uptake occurred between 4 and 12 h of growth. There was no indication that a manganese(III) complex appeared or accumulated over time. The cells that were harvested after 18 h and either treated with ethanol 70% (v/v) or sonicated resulted in no further change in the spectrum. The purpose of the ethanol treatment and the sonication process was to determine whether the oxidation of manganese(II) to manganese(III) occurred during bacterial death in the pro-oxidizing environment and whether the necessary stabilizing agents were present within the bacterial cells. The result suggested that neither the ethanol treatment nor the sonication process used to simulate bacterial death conditions showed any characteristics of manganese(III) formation.

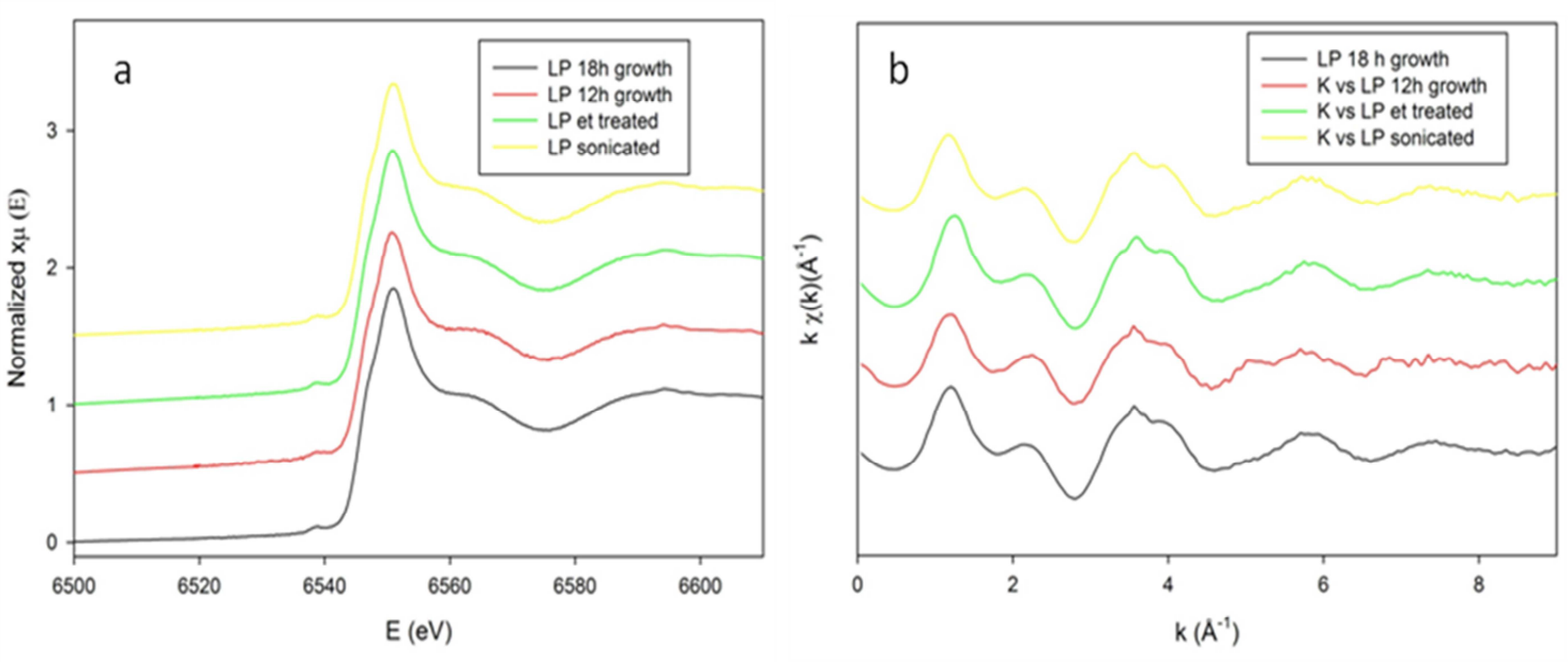

Figure 6-8 (a) XANES spectra and (b) EXAFS spectra of *L. paracasei* harvested after 12 h (red) or 18 h (black) of growth in MRS medium. *L. paracasei* harvested after 18 h and then treated with ethanol (green). *L. paracasei* harvested after 18 h and then sonicated (yellow).

Figure 6-8(b) compares the EXAFS spectra of the dead *L. paracasei* cells (by ethanol treatment or sonication) with the spectra of the cells harvested after 12 and 18h. The best fit for *L. paracasei* incubated for 18 hin a medium containing 224 mM manganese(II) was to the spectrum of a sum of 0.695 manganese(II) phosphate + 0.295 manganese(II) acetate, which summed to 1; this had an R factor of less than 0.002. An R factor value of less than 0.05 is considered to be a good fit (refer Appendix B4).

After collecting background information using XAS in relation to the manganese in the *L. paracasei* bacterial system, the focus was to determine whether the pro-oxidative environment [storage of bacteria under high water activity conditions (0.52 a_w)] leads to increased bacterial death because of the conversion from manganese(II) to manganese(III). Another part of the study was to find the possible causes for the observed differences in the ESR spectra of the freeze-dried, spray-dried and fluidized-bed-dried powders stored at 0.52 a_w, as observed in Chapter 4.

The XANES and EXAFS spectra of the freeze-dried, spray-dried and fluidized-bed-dried powders stored at all three different water activities are compared in Figure 6-9. The sample spectrum was similar to that of the manganese(II) model compound but not to that of the manganese(III) model compound, which indicated the presence of manganese in the system and no difference in the manganese speciation during storage.

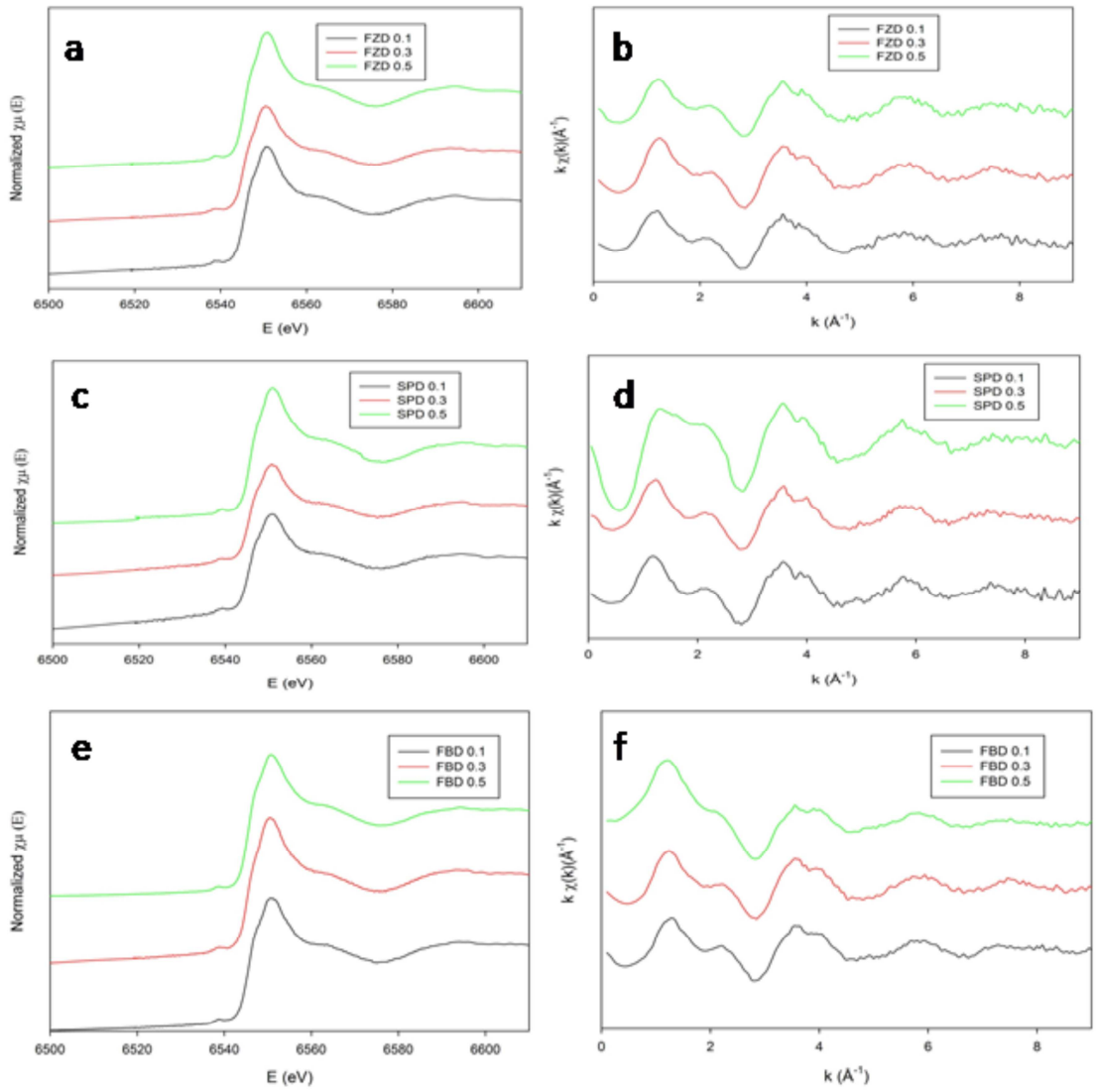

Figure 6-9 (a) XANES and (b) EXAFS spectra of freeze-dried *L. paracasei* in (FZD 0.1) 0.11 a_w, (FZD 0.3) 0.33 a_w and (FZD 0.5) 0.52 a_w; (c) XANES and (d) EXAFS spectra of spray-dried *L. paracasei* in (SPD 0.1) 0.11 a_w, (SPD 0.3) 0.33 a_w and (SPD 0.5) 0.52 a_w; (e) XANES and (f) EXAFS spectra of fluidized-bed-dried *L. paracasei* in (FBD 0.1) 0.11 a_w, (SPD 0.3) 0.33 a_w and (SPD 0.5) 0.52 a_w.

It has been reported previously that *L. plantarum* takes up manganese in the manganese(II) oxidation state (Archibald and Fridovich 1982a; Barnese et al. 2008; Barnese et al. 2012),

but the studies lacked *in situ* characterization. After establishing the presence of relatively high amounts of manganese in *L. paracasei* using ICP–OES/ MS and EDS, the next step was the use of XFM to monitor the changes in manganese during storage, to provide valuable information in relation to bacterial viability in dried powders. The samples stored at a_w 0.52 had different ESR spectra (hyperfine splitting) from the samples stored at lower water activity (a_w 0.33, 0.11), which had broad spectra that were characteristic of manganese phosphate.

Overall, an important aspect of this work was evidence that the presence of free manganese along with water in the freeze-dried and spray-dried powders stored at a_w 0.52 resulted in hyperfine splitting. Similar hyperfine splitting of manganese in the presence of water had been reported previously (Puskin et al. 1980). This hyperfine splitting may possibly have arisen as a result of water surrounding the manganese being released from the dead bacteria. However, such hyperfine splitting was concealed in the case of the fluidized-bed-dried powder stored at a_w 0.52, probably because of the rigid structure of the fat, the lower protein hydration (as previously observed in Fourier transform infrared spectra) and the different crystal form (as observed by X-ray diffraction).

TEM of the a_w 0.11 and a_w 0.52 freeze-dried samples suggested increased molecular mobility when stored under higher water activity conditions. The TEM results (in which an increased molecular disorder was seen in samples stored at a_w 0.52 compared with samples stored at a_w 0.11) supported the Fourier transform infrared and Raman spectroscopic results reported in Chapter 4. Moreover, damage to the bacterial cell membrane was observed in the samples stored at a_w 0.52, whereas no cell membrane damage was observed in the samples stored at a_w 0.11. An electron-dense substance was observed in the samples stored

at low water activity (a_w 0.11). However, there was loss of the manganese deposit from the samples stored at higher water activity (a_w 0.52). This shed light on the results from this study. For live bacteria, the glutaraldehyde fixation step was able to retain the manganese; however, for dead cells stored at high water activity (a_w 0.52), the manganese leached out either during the TEM sample preparation step or via the ruptured cell membrane as a result of membrane lipid oxidation during bacterial death.

Manganese is known to possess pro-and anti-oxidative properties, and understanding changes in the manganese oxidation state is considered to provide some further insight into the mechanisms of bacterial death. Manganese(III) has previously been reported to cause oxidative damage to neuron cells (Gunter et al. 2006). Thus, determination of the oxidation state of manganese was considered to provide valuable information on the loss of viability of the bacteria stored at higher water activity. ESR was limited to visualizing only the manganese(II) oxidation state at liquid nitrogen temperature; manganese(III) detection required a very low temperature of 10 K, which was not available at the ESR facility. Therefore, to understand the oxidation state of the manganese bodies, EELS combined with TEM was carried out. However, EELS could not detect the manganese core loss peak and a further attempt using EDS to detect manganese in TEM sections was unsuccessful. The reason why the electron-dense manganese particles in the bacteria could not be detected is unclear. It may be that the nanosize of the particles was beyond the spatial resolution of the CM-120 EELS technique and the JEOL-1400 EDS technique.

In view of the relatively high concentration of manganese in lactobacilli and the pro- and anti-oxidative properties of manganese (HaMai and Bondy, 2004; Jaramillo et al. 2012), it was important to better understand the oxidation state, the coordination number and the

ligands of the manganese in bacteria embedded in a dairy matrix and their change upon storage in controlled water activity environments. It appeared to be possible to characterize the changes in manganese within bacteria using XANES (Gunter et al. 2006; Haumann et al. 2005; Smolentsev et al. 2009), along with model compounds containing manganese(II) phosphate, manganese(II) carbonate, manganese(II) hydrogen phosphate and manganese(III) acetate, which are supposed to provide a pool of samples that might further aid in modelling the *in vivo* speciation of manganese.

Synchrotron XAS was employed to determine the changes that occurred in manganese bodies during storage of the bacteria under high water activity conditions (0.52 a_w), when there was an increased loss of bacterial viability compared with storage under lower water activity conditions (0.11 a_w).

The XANES data obtained from the *L. paracasei* clearly demonstrated that intracellular manganese in probiotic *Lactobacillus* sp. is predominantly taken up as manganese(II) *in situ*, and both the EXAFS data and the XANES data strongly favoured a mixture of manganese(II) phosphate and manganese(II) acetate as the binding ions. A manganese(II) phosphate and manganese(II) acetate mixture in a ratio of 70:30 resulted in a better R factor that was found with other possible combinations. The presence of manganese in the form of manganese phosphate was previously suggested, when grown in a manganese-rich medium (Archibald and Fridovich 1981b, 1981a, 1982a; Barnese et al. 2008, 2012; Puskin et al. 1980), utilizing ESR spectroscopy. The *in situ* ESR spectroscopy of bacteria and the XAS results obtained from the present study reconfirmed that phosphate ions are attached to manganese in *L. paracasei*.

Manganese(II) acetate standard was used in the current study with the idea that it would be a proxy for protein/peptide interactions with manganese. Manganese-associated proteins and peptides have been reported to provide protection in radiation-resistant *D. radiodurans* (Tabares and Un, 2013; Daly et al. 2006). However, as manganese possesses pro- and anti-oxidative properties (HaMai and Bondy 2004), association of manganese with phosphate and peptides when grown in a manganese-rich medium may prevent manganese sequestering with vital cellular proteins within the cells. Manganese phosphate (Archibald and Fridovich 1982a; Barnese et al. 2008, 2012) has previously been reported to possess free-radical-scavenging activity, and is possibly the only mode of defence against free radicals in *Lactobacillus* sp. in the absence of manganese superoxide dismutase (Archibald and Fridovich 1981a, 1981b).

The possibility of intracellular manganese forming complexes differently within different *Lactobacillus* sp., under live and dead conditions, during storage or in different growth phases, cannot be ignored. However, the present result suggested that the bacteria are more likely to remain in the same state and form. There were no indications of any difference in the manganese oxidation state that could be linked to possible bacterial death, during ethanol treatment or sonication, which were used to simulate bacterial death conditions. Also, from the spectra, there was no indication of the presence of manganese(III) complexes. Moreover, manganese(III) formation in a more pro-oxidative environment, such as storage under higher water activity conditions, could not be established. If manganese(III) is formed and is rapidly reduced to manganese(II) before measurable accumulation occurs, the nature of the damage would probably be difficult to distinguish from other cell damage mechanisms. However, there is also the possibility that, as

manganese(II) is present in higher amounts, this might disguise the presence of small traces of manganese(III) (Fernando et al. 2010; Gunter et al. 2006). A technique such as μ-XANES using X-ray microspectroscopy may be useful in determining speciation [the presence of manganese(III)] *in situ.*

In situ X-ray crystallography results (G B Jameson, personal communication, December 2012) for manganese hyper-accumulation in *L. paracasei* did not reveal crystalline manganese phosphate. Manganese present in the bacteria may therefore occur predominantly in soluble form, similar to that reported in foliar manganese in manganese hyper-accumulators in plant cells (Bidwell et al. 2002; Fernando et al. 2006).

In a separate study (refer Appendix Figure B1 and B2), XANES and EXAFS spectrum of *Lactobacillus plantarum* 299v (a probiotic strain harvested after 18h growth) was analysed. No difference between the spectra of the *L. paracasei* and *L. plantarum* cells could be observed. Moreover, freeze dried samples stored at 4, 20 and 40ºC did not any show differerence.

6.4 Conclusions

Most past researchers (Watanabe et al. 2012; Archibald and Fridovich 1982a; Barnese et al. 2008, 2012) have focused on *L. plantarum* and the presence of manganese deposits in this bacterium. The present study provided strong evidence that the binding of manganese in probiotic *L. paracasei* and *L. plantarum* may possibly be a mixture of manganese phosphate and manganese acetate, with the acetate acting as a proxy for protein interactions with manganese. The recent focus in the field of probiotics has been to incorporate probiotics into ambient long shelf life products (Chavez and Ledeboer, 2007). The addition

of manganese to the growth medium is known to enhance the stability of lactobacilli (Watanabe et al. 2012). However, less is known about how this accumulated manganese might behave during storage. Manganese having pro- and anti-oxidative properties raises the question of the safety of probiotic food products with long shelf lives. In the present study, XAS showed convincingly that the manganese present in *L. paracasei* is predominantly manganese(II) with phosphate and acetate being the binding ligands. It should be mentioned that no visible changes in the speciation of manganese could be observed in *L. paracasei* during ambient storage at water activities of 0.11, 0.33 and 0.52. There were no differences during various stages of growth or during sonication or ethanol treatment to induce cell death. Future studies, using XFM combined with XANES to determine the presence of manganese(III), are required to determine the formation of manganese(III) in bacterial cells during the cell death process. Moreover, it would also be of interest to explore the various levels of manganese accumulated by *L. paracasei* during various stages of growth and to look for possible correlations between storage stability and manganese levels in bacteria.

Chapter 7 Overall discussion, conclusions and recommendations for future work

7.1 Discussion

Probiotic bacteria have gained tremendous attention due to their health beneficial effects. For probiotic bacteria consumed as a part of food, it is generally agreed that 10^6 CFU (colony-forming units) of viable cells per gram of food product need to be present at the end of shelf life to confer a health benefit (Dave and Shah, 1997; Kebary, 1996; Lee and Salminen, 1995; Rybka and Kailasapathy, 1995). During International Scientific association for probiotics and prebiotics (ISSAP) meeting held on 23rd October 2013, several clinical and scientific experts, suggested that the probiotic framework must only include microbial species that have been shown to provide health beneficial effect in well conducted controlled human studies, and manufacturers using probiotics in food or food supplement with a specific health claim must provide proof delivery of viable probiotics at efficacious dose at the end of shelf life (Hill et al. 2014). To stabilize probiotic bacteria for long term storage, a suitable stabilization technology, together with an appropriate drying method, matrix constituents and matrix architecture, is required. Therefore, the overall aim of this thesis was to get a better understanding of the factors influencing the storage stability of the probiotic bacteria. The encapsulation of bacteria in protective matrix capable of minimizing the loss in the bacterial viability during storage is considered to have promising potential for the application of probiotics in dried products.

The objective of the preliminary study was to gain some fundamental understanding of the role of matrix components, such as fat, protein and carbohydrate, on the viability of the *L.*

paracasei during storage at temperature (4, 25 and 37ºC). WMP, SMP or MPI was used to simulate the protective effect of matrix containing milk fat+ milk protein + lactose, milk protein + lactose and only milk protein. The *L. paracasei* harvested from the stationary phase were used in the study, as they demonstrate greater stress resistance than log phase cells (Chapter 5). The probiotic lactobacilli embedded in the WMP matrix, using fluidized bed drying technique, provided better protection compared to SMP and MPI matrix. Upon storage, as expected, with an increase in water activity of the sample a decrease in bacterial viability was observed, consistent with the findings of the previous researchers (Ananta et al. 2005; Miao et al. 2008). Similarly, an increase in the storage temperature resulted in an increase in the bacterial death. Moreover, the difference in the storage stability of the powders produced by fluidized bed drying was also found to be influenced by the crystallization of lactose in the samples, which further reinforced the importance of glassy state responsible for preventing cell death, as reported by other researchers in the past (Miao et al. 2008). The protective effect of the fat was observed in the WMP samples stored at 37°C, where a 2.5 $\log_{10}$ reduction was observed during the first four weeks of storage. In contrast, the matrix comprising of SMP and MPI provided limited protection when stored at 37ºC, resulting in complete loss in viability in the SMP and MPI samples. The importance of fat in providing greater viability on the *L. paracasei* was also consistent with the results of previous reports where the presence of fat in the matrix was found to improve the storage stability of encapsulated probiotic bacteria (Hou et al. 2003; Lahtinen et al. 2007). The confocal laser scanning microscopy study provided an insight into the location of bacteria in fluidized bed dried powder; where bacteria were found just under the surface of the matrix, mainly as aggregated clumps.

With these preliminary observations, it was important to explore the following questions.

Does the drying technique have an influence on viability? Could choosing different techniques provide new insights into the changes in matrix that might be linked to the bacterial viability?

Does the presence of fat in the encapsulating matrix improve the viability of the bacteria? What are the suitable techniques to study the changes in the bacterial matrix during storage?

Does crystallization of lactose affect the bacterial viability?

Is water activity or water in the dried sample important?

Is there a possibility to improve the bacterial viability by controlling the water activity of the samples?

How does the powder structure relate with the bacterial viability?

To answer these questions, initially the impact of drying methods on the survival of *L. paracasei* in a whole milk powder matrix during storage at 25 °C was explored. As expected, for all drying methods (freeze drying, spray drying and fluidized bed drying) used, the water activity during storage of the dried powders was important. A higher water activity (0.5 a_w) at 25ºC during storage led to an increased loss of bacterial viability. There lied the need to understand how the differences in storage life between the powders produced by different drying methods arise.

Although the initial moisture contents of the powders differed markedly, this did not seem to be an important in the long term viability of the *L. paracasei*. Although the powders had initial moisture contents ranging from 1.7 to 7.2 wt%, these moisture contents did not reflect those after storage under controlled humidity.

Previous studies have shown the importance of the moisture content in the viability of bacteria in powders during storage, with increasing in moisture content leading to a rapid loss of bacterial viability (Gunning et al. 1995; Hsiao et al. 2004; Miao et al. 2008). The moisture contents of the powders stored at 0.11 a_w were in the range 1.7−2.6%; at this low level, the bacterial viability was retained for long periods (115 days). However, it would be difficult to maintain such low humidity storage environments in the commercial use of such products. At 0.33 a_w, the moisture contents of the stored powders increased to 4.8–5.4%, which affected the bacterial viability; small differences in moisture content led to large differences in storage life.

At higher water activity (a_w 0.52), the moisture content continued to increase during storage, with a corresponding reduction in the viable bacterial cell counts. During storage of the powders at 0.52 a_w, crystallization of lactose occurred, i.e. β-lactose for the spray dried and freeze dried powders and α-lactose monohydrate for the fluidized-bed-dried powder, as indicated by X-ray diffraction and FTIR spectroscopy. The water strongly bound as water of crystallization was not removed during the moisture content estimation (102 ± 2 ^oC for 3 h). Equilibration of the powders at 0.52a_w led to a shorter storage life of viable bacteria, possibly due to the loss of the stabilizing glassy state with the formation of crystalline lactose. The glassy state provides a matrix that limits molecular mobility and therefore limits

the exchange of oxygen. This may possibly limit the deteriorative oxidative reactions that lead to bacterial membrane lipid oxidation and death (Yao et al. 2008).

The differences in the moisture content after storage under controlled humidity environments between the powders produced by different drying methods were probably due to the morphology, including the porosity and the structure, of the powders. The external morphologies were clearly different, with spray drying producing largely free spherical particles of 20–100 µm diameter, fluidized bed drying producing larger agglomerated powders of 100–700 µm and freeze drying producing large plates. Fluidized bed drying and spray drying produced similar porosities, i.e. around 38% connected porosity. In contrast, freeze drying produced 71% connected porosity. The moisture content after equilibration at controlled humidity was the highest for the freeze dried material and the lowest for the fluidized-bed-dried material. It is likely that water was primarily absorbed initially to the powder surfaces. The surface area for water absorption depends on both the particle size and the porosity. The fluidized-bed-dried powder had the lowest surface area, which was probably why it had the lowest moisture content after storage in a controlled humidity environment. The freeze dried material had the highest connected porosity, providing a large internal surface area for water absorption, which may have been the reason for its high moisture content after storage in a controlled humidity environment.

The structures of the whole milk powder and *L. paracasei* mixtures varied upon storage in a controlled water activity environment (0.11, 0.33 and 0.52 a_w). At the highest water activity ($0.52a_w$), all the powders (freeze dried, spray dried and fluidized bed dried powders) showed crystallization. This was evident as the development of Bragg peaks in X-ray diffraction, crystalline lactose vibrational absorbances in FTIR spectroscopy and a decrease in

the moisture that was released by heating at 102 °C. However, the bacteria in all of these powders had very poor storage stability. Therefore, even though some of the water was tied up as water of crystallization, this did not improve the stability of the bacteria.

As the differences between the different drying techniques were apparent after storage at 0.33 a_w, the differences between the powders under these conditions may reveal aspects of structure that affect bacterial storage stability. X-ray diffraction showed no differences, as all these powders appeared to be X-ray amorphous. However, FT-IR PCA identified notable differences in molecular mobility and indicators of hydration of protein and lactose between less storage-stable powder (spray dried and freeze dried) and more storage-stable powder (fluidized bed dried).

Higher water activity conditions have always been found to be associated with bacterial death. However, the presence of high amount of water in the matrix also creates interferences, which poses difficulty in studying FT-IR spectrum. However, Raman spectroscopy provided the added advantage to studying the samples without interfering water signals. Apart from the crystallization of the samples observed in the samples stored at 0.52 a_w, Raman spectroscopy was also able to identify an increased molecular mobility of fat in the samples stored at high water activity conditions (0.52 a_w). The Raman spectroscopy result indicated that the fluidized bed dried powder had a less mobile fat compared to spray dried and freeze dried powders.

Molecular mobility has generally been associated with bacterial death; a classic example is the crystallization of lactose in the samples and simultaneous loss in bacterial viability (Miao et al. 2008), which indirectly suggest molecular mobility in the system. A possible rea-

son is that a increased molecular mobility results in increased rate of oxidative reactions, within the powder resulting in lower bacterial viability. Therefore, ^{13}C solid state NMR was carried out to assess the molecular mobility in the system. The ^{13}C solid state NMR spectrum of the samples suggested that the fluidized bed dried powder was most stable.

The use of different spectroscopic techniques to study the changes in the matrix which could be correlated with bacterial viability was carried out to ensure comprehensive characterization of the embedding matrix system, and thereafter study the changes during storage.

The ability of *L. paracasei* to maintain viability may be enhanced by the ability of the organism to accumulate manganese, which can act as a free radical scavenger. It has been observed that *Lactobacillus* sp. may accumulate large amounts of manganese, which provides protection to the bacteria against oxidative damage (Archibald and Fridovich, 1981a; Barnese et al. 2008, 2012; Daly et al. 2004). Recently, Barnese et al. (2012) demonstrated how manganese combats superoxide in the cellular system. An estimation of the relative concentration of low molecular weight ligands that bind to manganese was carried out, followed by an investigation on the reactivity of these manganese containing species with O^{2-}. The key finding from this study showed that under simulated physiological conditions of concentration and pH, both manganese phosphate and manganese carbonate are able to catalytically remove superoxide from solution. The level of manganese in freeze dried *L. paracasei,* using ICP-MS, was found to be 1.55 wt%, which was a high concentration for such a component (Archibald and Fridovich, 1981b). The contribution of manganese to enhancing the storage stability of probiotics in a milk powder matrix was therefore thought to be an important aspect to be considered in the next study.

X-ray fluorescent microscopy (XFM) was employed to determine the changes in the elemental composition of *L paracasei* during growth in MRS medium with or without manganese (manganese rich vs manganese deficient medium) and as a function of physiological growth state (early log vs. stationary phase). The results revealed that lower level of manganese accumulation occurred during early log phase of bacterial growth compared with the stationary phase cells. The lower level of manganese accumulation could be related to the loss in bacterial viability during the storage study. The results suggest bacteria harvested in early log phase, grown in MRS medium, may provide lower protection to the bacteria against oxidative stress and unsuitable for stabilization. The results are consistent with other reports which suggest the stationary phase cells are more robust for encapsulation (Corcoran et al. 2004). It may possibly be due to higher manganese accumulation occurring in the stationary phase cells. The present result also justifies the use of stationary phase harvested cells for all other experiments. Moreover, XFM results further validated that manganese was not present in the embedding matrix which could possibly interfere with manganese present in the bacteria. With the basic understanding of importance of manganese in *L. paracasei*, and the embedding matrix being deficient in manganese, it became obvious to explore the changes during storage, utilizing manganese as a tracer component. It was hypothesized that the changes that occur in the manganese ligands present in the sample stored at different water activity among the bacterial powders might provide new information about bacterial storage stability.

ESR spectroscopy which has the ability to detect species that have unpaired electrons, was used primarily for detecting the changes in the manganese speciation occurring in the bacteria during storage at low and higher relative humidity conditions. Further, it has an added

advantage of detecting *in-situ* manganese, without extraction or further processing which may change the manganese speciation. ESR spectroscopy provided useful insights; the samples stored at low water activity, primarily 0.1 a_w and 0.3 a_w had a characteristic broad spectrum representing manganese phosphate, which have been proposed to be present in the *Lactobacillus* sp. (Archibald and Duong, 1984). However, the sample stored at high water activity especially the freeze dried and the spray dried samples stored at 0.5 a_w had a characteristic splitting typical of hexaaquo species, while the fluidized bed dried powder stored at 0.5 a_w had characteristic broad spectrum. The ESR spectrum of the bacterial powders was readily obtained at room temperature. However, to acquire the ESR signal of the freshly harvested bacteria without the matrix, it was necessary to cool the sample to -160°C. The spectrum thus obtained had hyperfine splitting similar to that observed in spray dried and freeze dried powder stored at 0.5 a_w. ESR spectrum of the matrix without bacteria revealed no peaks and provided enough evidence that the ESR spectrum was originating from the bacteria and not from the matrix.

To validate that the origin of the spectrum was from only manganese and not from other transition elements, the g value of the ESR spectrum was determined. Each transition metal has a specific g value and the calculation of g value provides as a means to identify them. DPPH standard ran alongside to determine calculated g value was found to be “2.0”. The g value of 2.0 represents Manganese, as Mn (II), and has a characteristic ESR signal with the possibility of 8 line hyperfine splitting from the I= 7/2 55Mn nucleus. The changes observed in the in the ESR spectrum led us to explore the possibility of visualizing the manganese deposits and the changes that might be further monitored to determine the causes of increased bacterial death during storage at higher water activity conditions.

Transmission electron microscopy, used to study the difference between the low (0.11 a_w) and high water activity (0.52 a_w) stored samples, revealed mobility of fat in the samples stored at 0.52 a_w. This finding was consistant with FT-IR and Raman spectroscopic results, where an increased C-H stretching was observed in the samples stored at 0.52 a_w , while no such mobility was observed in the TEM images of the samples stored at low water activity (0.11 a_w). Moreover, the damage of the bacterial cell membrane was observed in the samples stored at high relative humidity, while no cell membrane damage was observed in the samples stored at low relative humidity. Electron dense material was observed in the samples stored at lower water activity conditions (0.11 a_w). However, there was a loss in the manganese deposits in the higher water activity (0.52 a_w) stored sample. A possible explanation might be the bacterial death results in the lowering of the bacterial cytosolic pH resulting in the solubilization of manganese. The result of a study carried by Archibald and Duong (1984) was able to explain the reason for the observed difference. Their study highlighted that formaldehyde (1%v/v) or glutaraldehyde (5% v/v) was able to seal the cells completely preventing the loss of manganese. While, in toluene treated cells, which ruptures the cell membrane (simulating cell death conditions), it was observed that the addition of glutaraldehyde or formaldehyde did not prevent the manganese exit from the cells. This shed light into the observed results; in the case of live bacteria, glutaraldehyde fixation step was able to retain manganese, but in the case of dead cells, upon storage at higher water activity conditions, the manganese leached out during the TEM sample preparation step, through the ruptured cell membrane as a result of membrane lipid oxidation during bacterial death.

Manganese has been known to possess pro and antioxidant properties, and understanding of the changes in the manganese oxidation state was considered to provide some further insight into the bacterial death mechanism. Mn^{3+} has been previously reported to cause oxidative damage to the neuron cells (Gunter et al 2006). Determination of oxidation state of manganese may provide rational for the loss in viability of bacteria stored at higher RH. ESR was only limited to looking at the Mn^{2+} oxidation state at liquid nitrogen temperature and Mn^{3+} detection required very low temperature of 10 K which was not available in the ESR facility. Therefore, to understand the oxidation state of the manganese bodies, Electron Energy loss microscopy (EELS) attached with the TEM was used. The EELS spectrum acquired in a range from 400 to 900 eV, revealed only O-K- edge (532 eV), while Mn L2 and L3 edges at 640 eV and 651 eV could not be observed in this spectrum. The reason of not detecting the electron dense manganese particles in bacteria is not clear. One possibility is that biological samples are electron irradiation sensitive and could not tolerate long-time exposure of electron radiation when applying EDS or EELS even though they were observed under 120KV. The other aspect might be that the nanosize of the particles was beyond the spatial resolution of CM120 EELS and JEOL- 1400 EDS techniques.

In view of the relatively high concentration of manganese in lactobacilli, and manganese having pro and antioxidant properties (HaMai and Bondy, 2004; Jaramillo et al. 2012), it was of interest to better understand the oxidation state, coordination number and ligands of the manganese in the bacteria in different encapsulating and relative humidity environments. It appeared possible to characterize the changes of manganese within bacteria using XANES (Gunter et al. 2006; Haumann et al. 2005; Smolentsev et al. 2009) alongside model

compounds, Mn (II) phosphate, Mn (II) carbonate, Mn (II) hydrogen phosphate and Mn (III) acetate.

Synchrotron X-ray absorption spectroscopy (XAS) represents a viable tool to study the oxidation state of elements *in situ*. Therefore, the goal of the present study was to employ Synchrotron XAS to determine the changes that occur in the manganese bodies during storage of bacteria at higher water activity (0.52 a_w) where there was an increased loss in bacterial viability compared to lower water activity (0.11 a_w) stored samples.

In this study, XANES data obtained from the *L. paracasei* 431 clearly demonstrated that intracellular manganese in probiotic *Lactobacillus* sp. was predominantly uptaken as Mn (II) *in-situ,* while both EXAFS and XANES data strongly favoured the presence of manganese phosphate in the samples as observed by previous researchers (Archibald and Duong, 1984; Barnese et al. 2012). XAS result was found to be consistant with the ESR results, which indicated the presence of manganese in the form of manganese phosphate. The possibility that intracellular manganese may infact be complexed differently within different *Lactobacillus* species, during live and dead conditions, during storage or during different growth phase, cannot be discarded. However, the results suggest that the bacteria are more likely to remain in the same state and form, during live and dead condition. There was no indication of any difference in the manganese oxidation state which can be linked with possible bacterial death, during ethanol treatment or sonication (simulated bacterial death condition). In case Mn (III) is formed and rapidly reduced to Mn(II) before measurable accumulation occurs, the nature of the damage would probably be difficult to distinguish from other cell damage mechanisms. However, there also exists the possibility that as Mn(II) is present in a higher amount, which might disguise the presence of small traces of Mn(III). A

technique such as μ- XANES using X-ray microspectroscopy may be useful in determining speciation (presence of Mn^{3+}) *in-situ*.

The differences in the Mn ESR signal of embedded *L .paracasei* between the freeze dried, spray dried and fluidized bed dried powders stored at 0.11 a_w and 0.52 a_w led us to explore further the changes causing the difference between fluidized bed dried powder compared with others. It was shown conclusively that the manganese present in the *L. paracasei* is predominantly present as Mn(II) phosphate. Therefore, it may be presumed that the observed ESR signal difference between the fluidized bed dried and spray and freeze dried bacteria stored at 52.5 RH was mainly due to the presence of water around the bacteria in the matrix, causing the hyperfine splitting. There is a possibility that the signal from the water may have been concealed in the fluidized bed powder which had a rigid structure, estimated by FT-IR, Raman and NMR spectroscopic data.

The recent focus in the field of probiotics has been to incorporate probiotics in ambient long shelf life products. Manganese has been found to provide protection to *Lactobacillus* sp. by scavenging free radicals. It has been previously established that the stress resistance of bacterial culture is dependent on the growth phase, for instance the bacteria that enters stationary growth phase develops a general stress resistance. The increased stress response of the stationary phase cells, compared with the bacteria in log phase was suggested to be probably due to the carbon starvation and exhaustion of available food resources, which trigger responses that allow cell survival (Van de Guchte et al. 2002). For example, probiotic *Lactobacillus rhamnosus* spray dried powders retained high viability (over 50% survival; $2.9x10^9$ CFU/g) when produced from stationary phase cells, while powders made from early log phase cultures exhibited only 14% survival (Corcoran et al. 2004) .

7.2 Conclusions and recommendation for future research

The WMP matrix combined with fluid bed drying provides the best protection to *L. paracasei* during storage under ambient conditions. This study also demonstrated that storage at high water activity condition (0.52 a_w) resulted in decrease in bacterial viability. One possible reason for the observed decrease in bacterial viability is the increase in molecular mobility observed in the powders stored at 0.52 a_w, which could lead to increased levels of oxidative reactions within the powder decreasing bacterial viability. As is the case with any research study, the presented results must be taken into consideration within the context of limitations. The initial research question was "to gain a fundamental understanding about the storage stability of ambient stored probiotic bacterial powders". The process of posing and answering particular research question, typically lead to more questions that were explored through further research.

The readers must understand the experiments carried out in the present thesis are of exploratory nature. Several analytical techniques were carried, which have previously found limited application in food research, the reason behind being the complex nature of the embedding matrix. The present thesis study is pictorially represented in Figure 7.1 and Figure 7.2. The key research findings obtained by employing spectroscopic techniques such as Fourier transform infrared spectroscopy, Raman spectroscopy, Helium pycnometry and Scanning electron microscopy on freeze dried, spray dried, and fluidized bed dried powders, were to find a relationship between matrix structure and matrix constituent influencing bacterial viability loss during storage at controlled water activity conditions of 0.11a_w, 0.33 a_w, and 0.52 a_w.

Higher porosity of powders retained higher water content during storage at higher relative humidity condition, resulting in increased viability loss during storage. Porosity of the structure has been found to be an important factor responsible for absorption of water during storage of the powders at controlled water activity conditions. Therefore, there is a need for further study to optimize the porosity levels of structure using different matrix constituents. Secondly, matrix constituents have been found to be involved in conferring protection to the bacteria, it is worthwhile to compare and contrast the porosity levels by changing the matrix composition and thereby improving the bacterial viability during storage.

Manganese accumulation in the bacteria was found to have an influence on the bacterial viability during storage. X-ray fluorescent microscopy to study the changes in bacterial metal ion concentration gradient during various stages of growth can further provide valuable information in relation to the robustness for cultures harvested during various phases of bacterial growth.

The above three key research areas could be further expanded using modeling to understand and optimize the process for ambient shelf stable probiotic powders, as represented in Figure 7.3.

It may be worthwhile to explore the genetic/proteomic aspect of the bacteria during storage. Genetic microarray analysis provides complete genetic profiling of the bacteria, and may provide the details of genes/ proteins expressed, during storage. The combination of the physical characterization together with genetic understanding would help in the long term stabilization of probiotic bacteria. A proposed genetic proteomic and XFM analysis approach suggested in Figure 7.4 might provide further useful information.

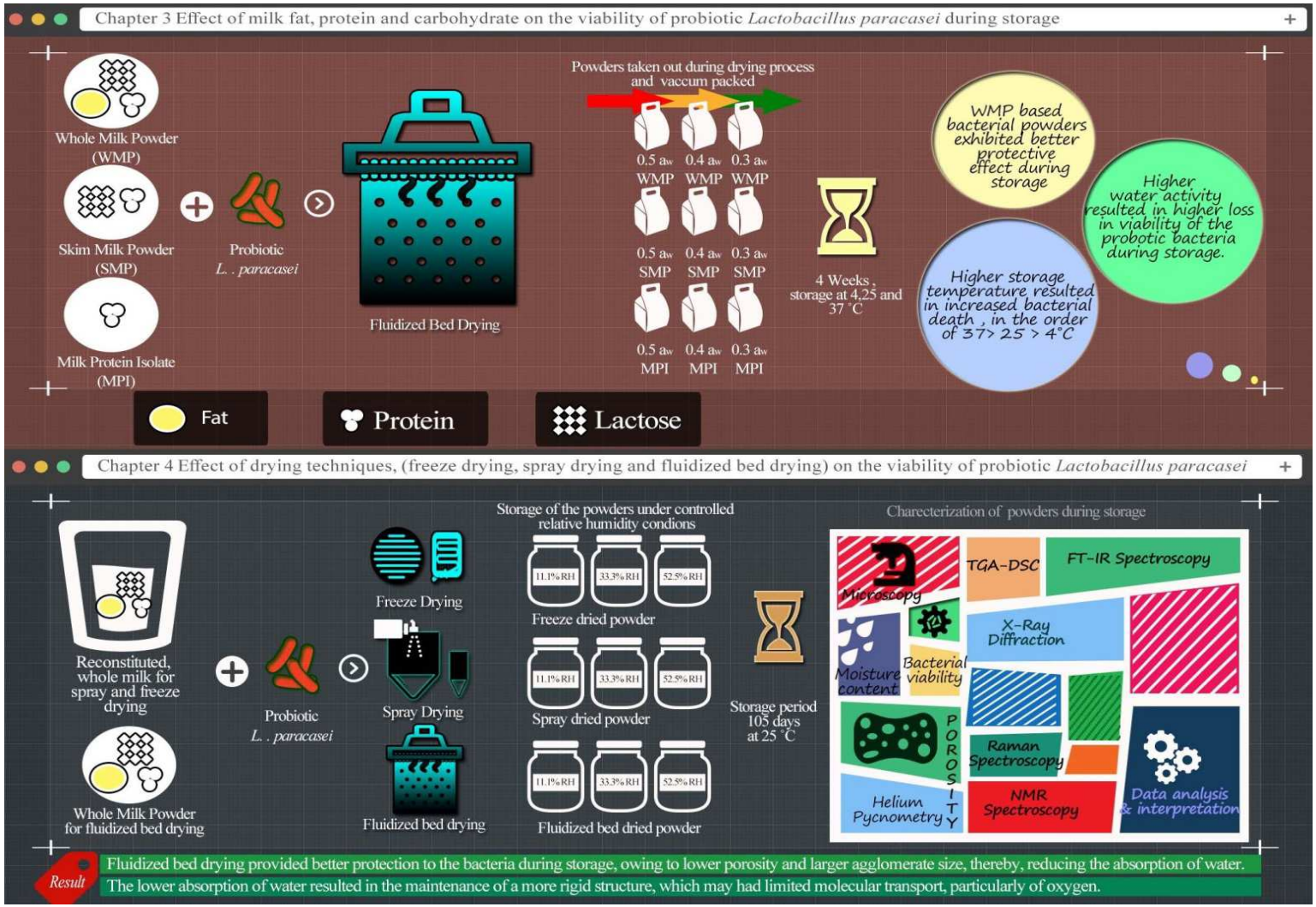

Figure 7-1 Pictorial summary of Chapter 3-4 and the key findings.

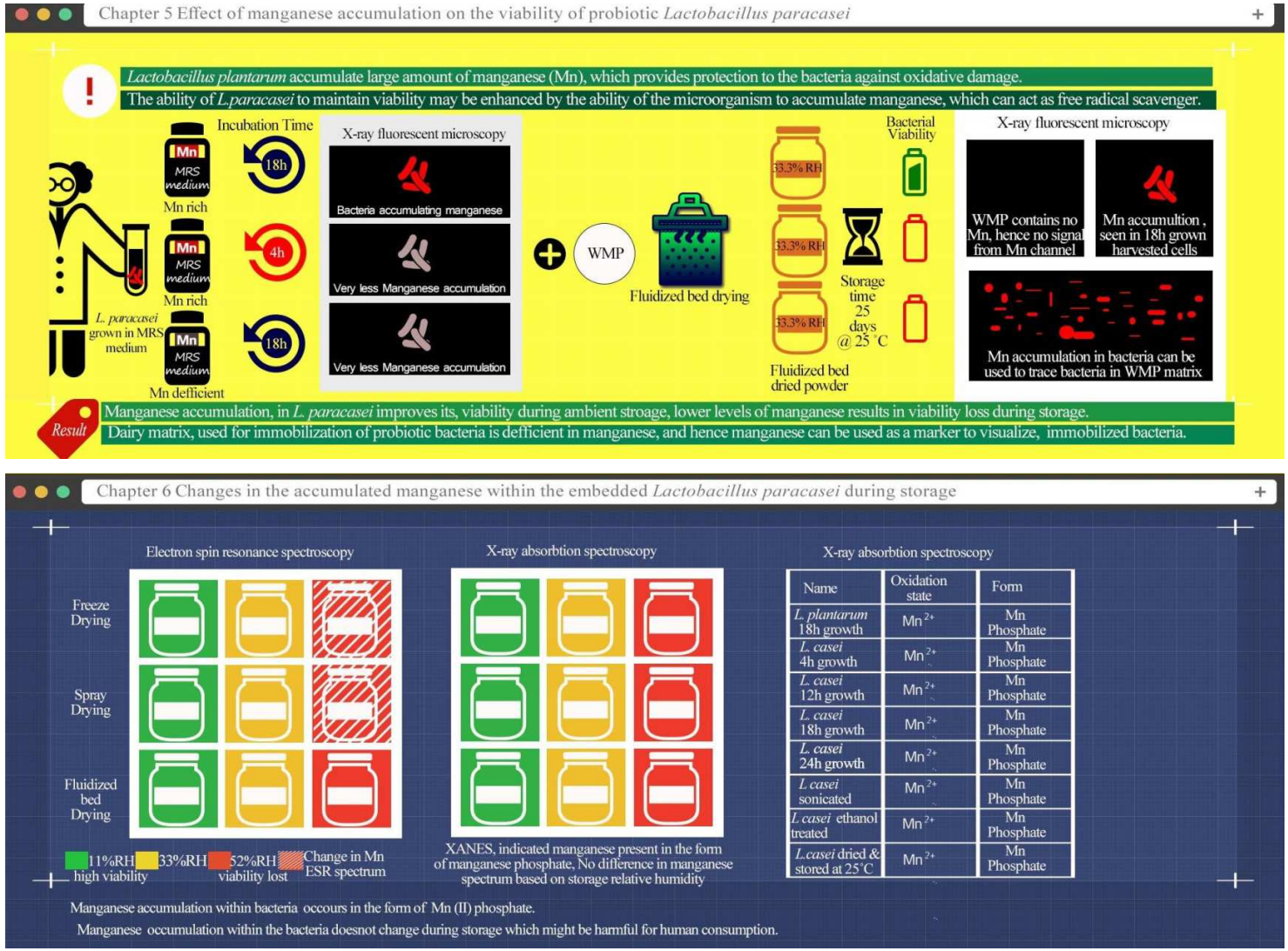

Figure 7-2 Pictorial summary of Chapter 5-6 and the key findings.

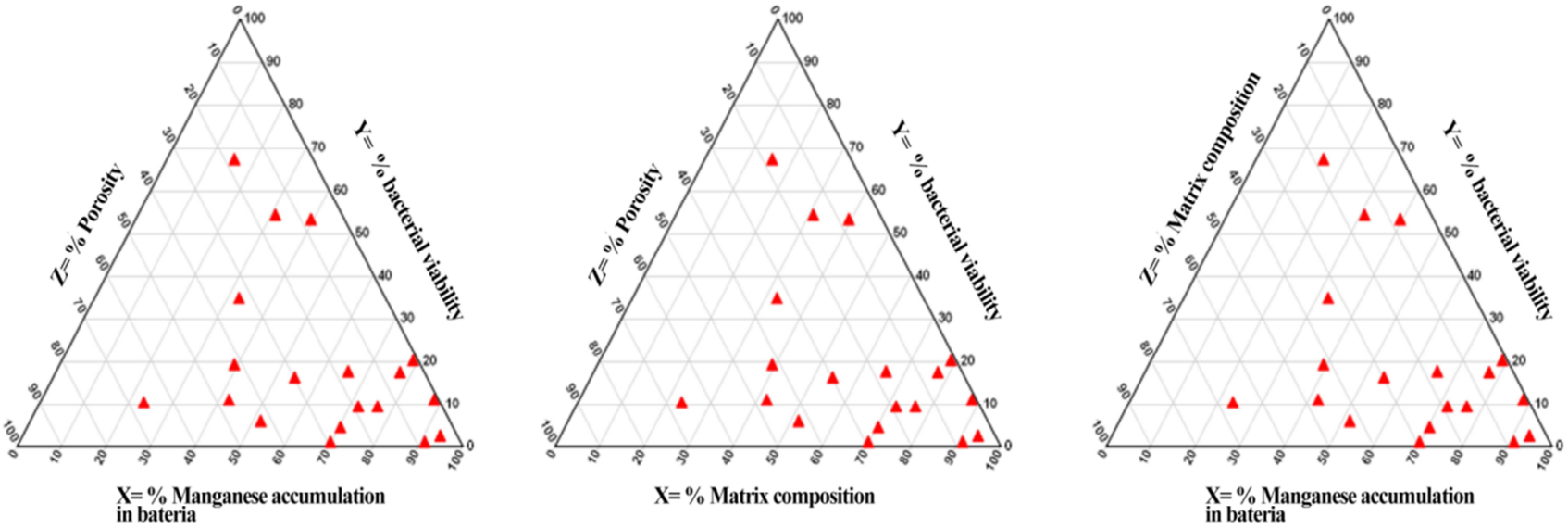

Figure 7-3 Hypothetical triangular graphs to show the possible future research possibility, using the present understanding of the thesis. Red triangles represent hypothetical data point.

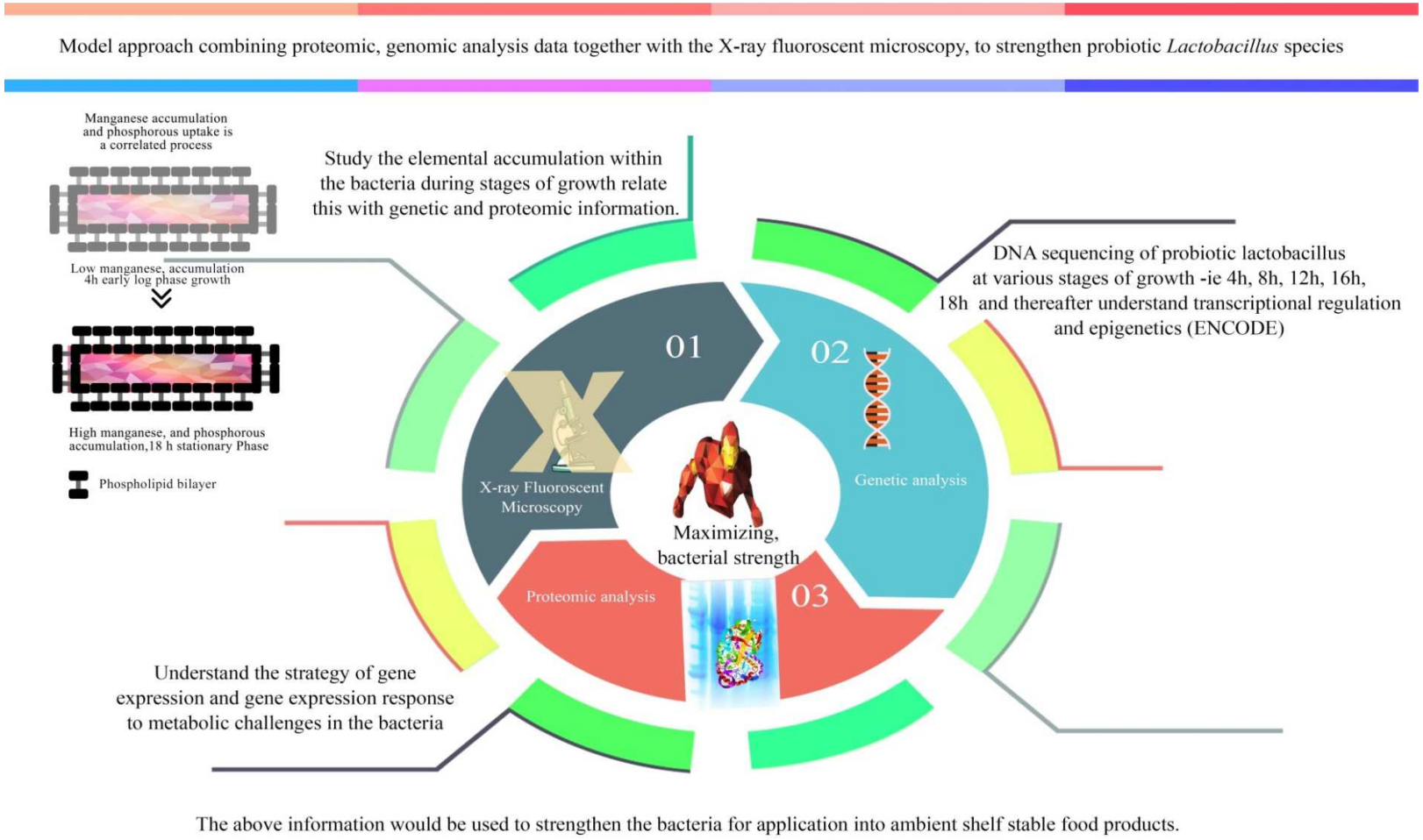

Figure 7-4 The proposed approach to improving the viability of probiotic *Lactobacillus* by gaining cell physiology information from genetic, proteomic and XFM analysis.

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APPENDIX A:

Table A-1 (a) Typical composition of MRS broth

Typical Formula*	gm/litre
Peptone	10.0
`Lab-Lemco' powder	8.0
Yeast extract	4.0
Glucose	20.0
Sorbitan mono-oleate	1 ml
Dipotassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Triammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Manganese sulphate $4H_2O$	0.05
pH 6.2 ± 0.2 @ 25°C	

Table A-1 (b) Composition of reconstituted MRS medium without added manganese

Typical Formula*	**gm/litre**
Peptone	10.0
`Lab-Lemco' powder	8.0
Yeast extract	4.0
Glucose	20.0
Sorbitan mono-oleate	1 ml
Dipotassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Triammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Manganese sulphate $4H_2O$	0
pH 6.2 ± 0.2 @ 25°C	

A-2 Analysis of X-ray diffraction patterns for the spray dried, freeze dried and fluidized bed matrix stored at water activity of 0.1, 0.3 and 0.5 a_w using 2D XRD.

Every crystal has a unique structure and a characteristic diffraction pattern when the X-rays are passed through it. X-ray diffraction was carried out on a Rigaku Micromax 007 X-ray generator, Rigaku RA axis IV ++ image plate detector and the data were examined using crystal clear software.

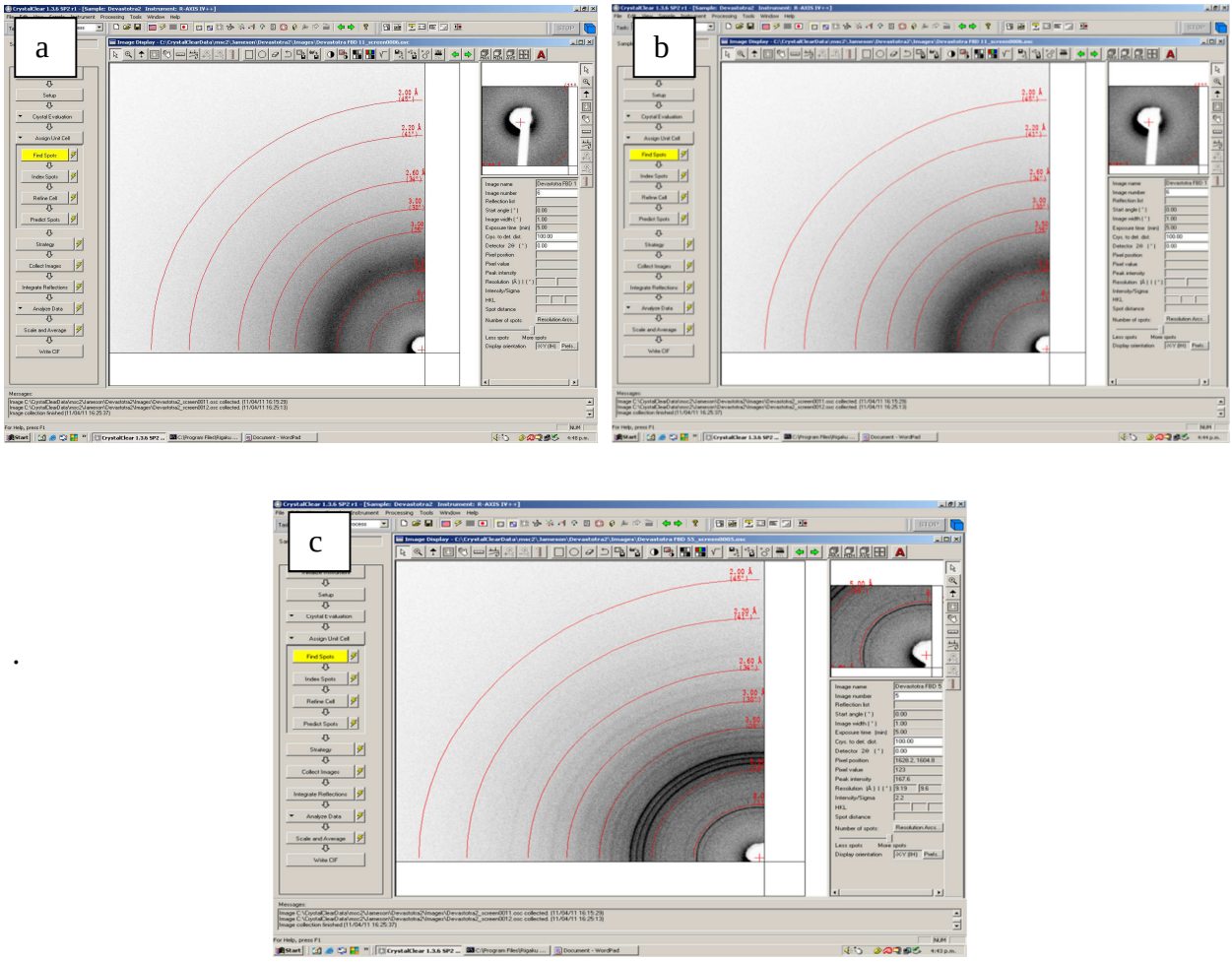

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Fig A-2 (a) Fluidized bed dried powder (a) 0.1 a_w (b) 0.3 a_w and (c) 0.5 a_w

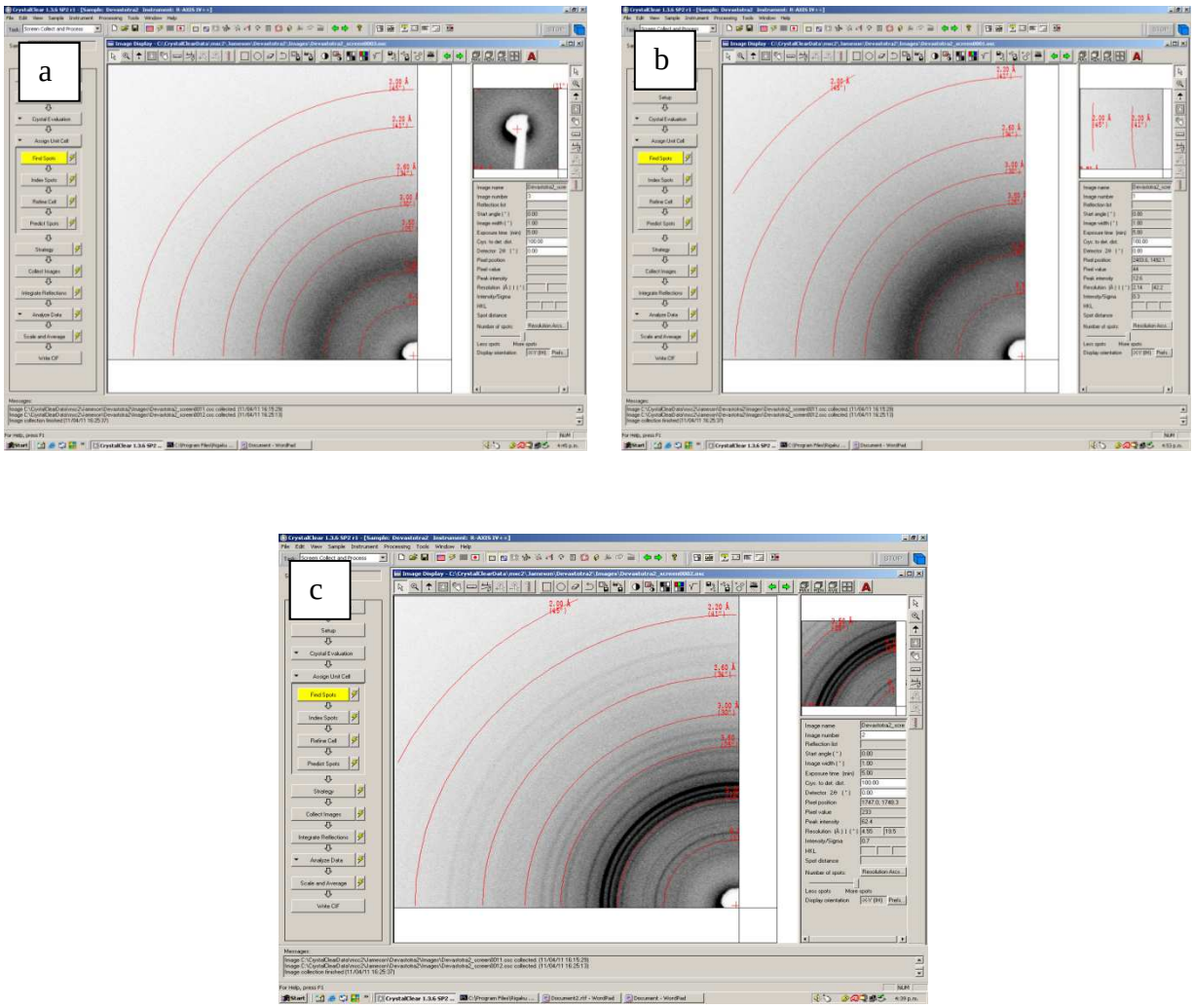


Fig A-2 (b) Spray dried powder (a) 0.1 a_w (b) 0.3 a_w and (c) 0.5 a_w

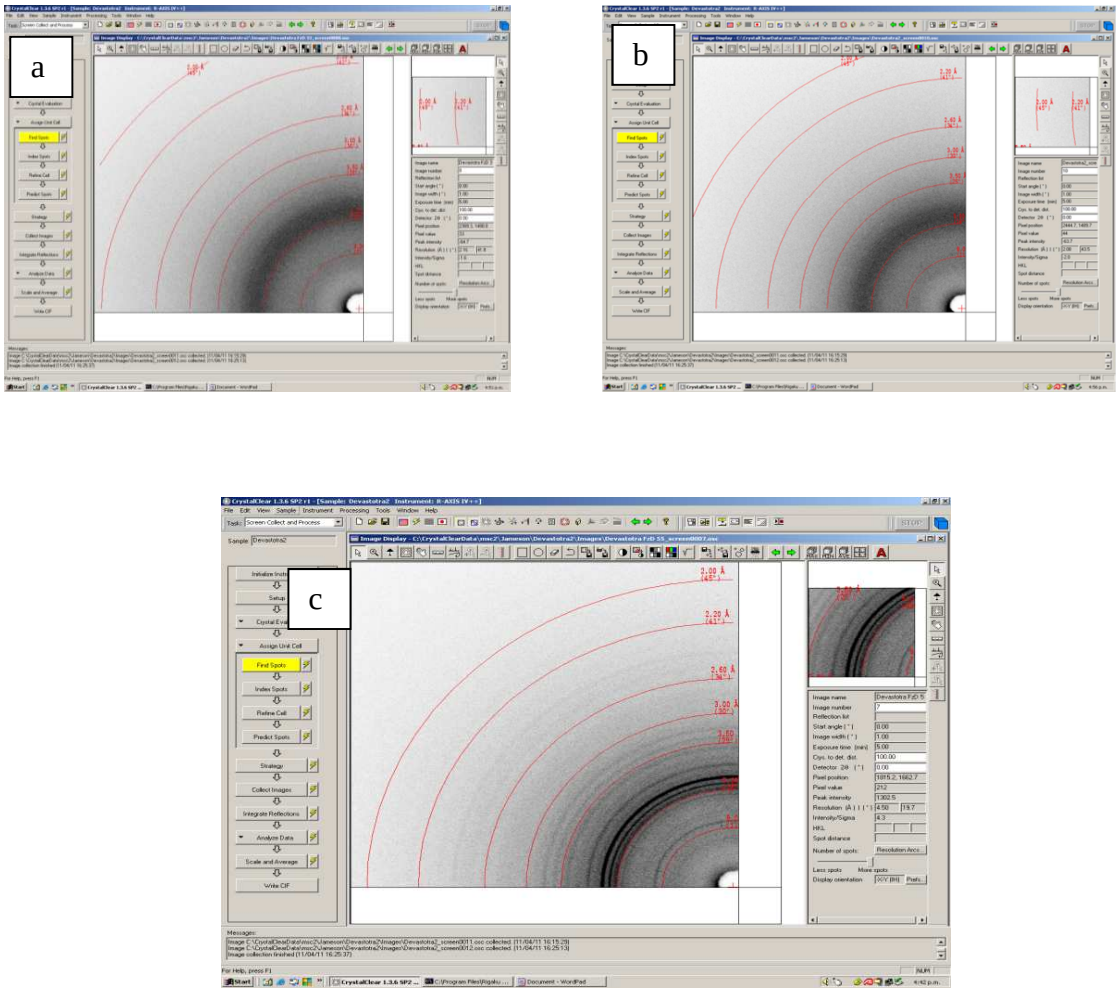


Fig A-2 (c) Freeze dried powder (a) 0.1 a_w (b) 0.3 a_w and (c) 0.5 a_w

Table A2 XRD diffractions peaks of freeze dried, spray dried and fluidized bed dried powders stored at 0.5 a_w

Fluidized bed powder 0.5 a_w		Freeze dried powder 0.5 a_w		Spray dried powder 0.5 a_w	
d	2θ	d	2θ	d	2θ
2.47	36.3	2.15	42.1	2.02	45
2.71	33.3	2.2	41	2.14	42.2
2.83	31.6	2.39	37.7	2.2	41
3.15	28.3	2.5	36	2.28	39.5
3.47	25.7	3.23	27.6	2.36	38.1
3.73	23.9	3.38	26.4	2.48	36.2
4.23	21	3.38	26.4	3.2	27.9
4.43	20	3.6	24.7	3.35	26.6
4.66	19	3.84	23.2	3.59	24.8
5.13	17.3	4.03	22	4.01	22.2
5.44	16.3	4.29	20.7	4.41	20.1
7.13	12.4	4.44	20	4.65	19.1
8.21	10.7	4.69	18.9	4.84	18.2
9.19	9.6	4.93	18	5.11	17.4
		5.18	17.1	6.2	14.3
		6.32	14	6.56	13.5
		7.35	12	7.14	12.4
				7.74	11.4

				8.71	10.2
				9.61	9.2

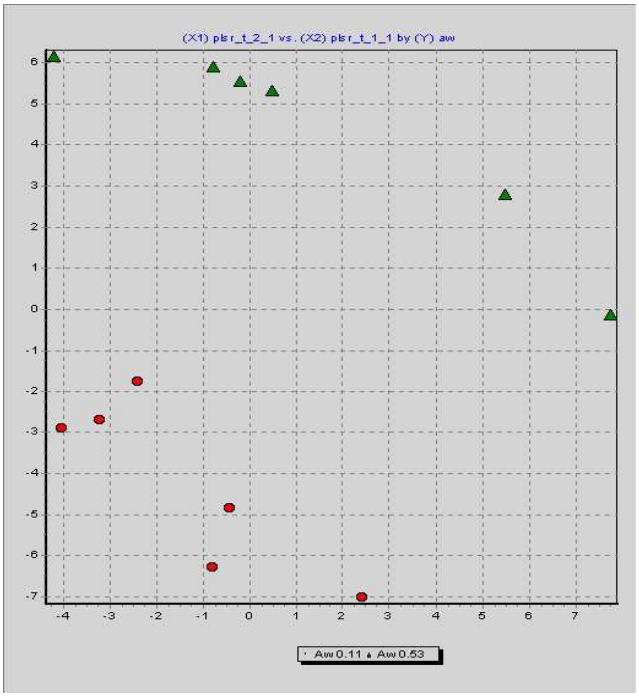


Figure A3 PLS-DA between aw 0.11 and aw 0.53 shows good separation. The regions which contribute to the separation are fat region.

Table A3 ^{13}C-NMR Charecterization of the molecular components of the lipid fraction of milk fat (Scano et al. 2011).

Peak	Compound	Carbon	Functional group	δ (ppm)
1	FFA	C1	OOC–CH–	178.04
2	Saturated FA in *sn*-1,3 of DAG	C1	–CH_2–OOC–CH_2–	173.35
3	Unsaturated FA in *sn*-1,3 of DAG	C1	–CH_2–OOC–CH_2–	173.32
4	FA in *sn*-1 (*sn*-3) of 1,2 (2,3) DAG	C1	–CH_2–OOC–CH_2–	173.18
5	FA in *sn*-2 of 1,2 (2,3) DAG	C1	–CH–OOC–CH_2–	173.08
6	Saturated FA in *sn*-1,3 of TAG	C1	–CH_2–OOC–CH_2–	172.79
7	Unsaturated FA in *sn*-1,3 of TAG	C1	–CH_2–OOC–CH_2–	172.7
8	Butyric FA in sn-1,3 of TAG	C1	–CH_2–OOC–CH_2–	172.6
9	Saturated FA in *sn*-2 of TAG	C1	–CH–OOC–CH_2–	172.41
10	Unsaturated FA in *sn*-2 of TAG	C1	–CH–OOC–CH_2–	172.38
11	Caproleic FA	C9	–CH=CH_2–	138.7
12	CLA	C12	–CH=CH	134.45
13	All n-3 FA	ω3	–CH=CH	131.66
14	VA FA	C12	–CH=CH	130.09-130.08
15	VA FA	C11	–CH=CH	130.00-129.98
16	Linoleic+linolenic FA	C13+C9	–CH=CH	129.89
17	MUFA	C10	–CH=CH	129.70-129.69
18	Lenoleic FA	C9	–CH=CH	129.51-129.49
19	MUFA	C9	–CH=CH	129.39-129.37
20	CLA	C10	–CH=CH	128.42
21	Linoleic FA	C13-C12	–CH=CH	127.97-127.92
22	Linoleic FA	C10	–CH=CH	127.77-127.76
23	Linoleic FA	C12	–CH=CH	127.59-127.58
24	Linoleic FA	C10	–CH=CH	127.46-127.44
25	All n-3 FA	ω4	–CH=CH	126.77
26	CLA	C11	–CH=CH	125.26
27	Caproleic FA	C10	–CH=CH_2–	114.05
28	Glycerol in 1,2/2,3 DAG		–CH–OOC–	71.85
29	Glycerol in TAG		–CH–OOC–	68.72
30	Glycerol in 1,3 DAG		HO–CH–$(CH_2)_2$	67.81

31	Glycerol in 1,3 DAG		–CH_2–OOC–	64.75
32	Glycerol in 1,2/2,3 DAG		HO–CH_2–CH–	62.02
33	Glycerol in TAG		–CH_2–OOC–	61.83
34	Glycerol in 1,2 DAG		–CH_2–OOC–	60.83
35	Butyric FA	C2	–OOC–CH_2–CH_2–	35.62
36	All FA except butyric in sn-2 of TAG	C2	–OOC–CH_2–CH_2–	33.92
37	All FA except butyric in sn-1,3 of TAG	C2	–OOC–CH_2–CH_2–	33.76
38	CLA	C13	–CH_2–CH=CH_2–	32.68
39	VA FA	C10,C13	–CH_2–CH=CH–	32.40-32.35
40	trans MUFA		–CH_2–CH=CH–	32.07-32.06
41	Saturated C > 10 FA	ω3	–CH_2–CH_2–CH_3–	31.74
42	Monounsaturated n-9 FA	ω3	–CH_2–CH_2–CH_3–	31.72
43	Capric FA C10:0	ω3	–CH_2–CH_2–CH_3–	31.67
44	Palmitoleic FA	ω3	–CH_2–CH_2–CH_3–	31.59
45	VA FA	ω3	–CH_2–CH_2–CH_3–	31.57
46	CLA	ω3	–CH_2–CH_2–CH_3–	31.56
47	Caprylic FA C8:0	ω3	–CH_2–CH_2–CH_3–	31.47
48	Linoleic FA	ω3	–CH_2–CH_2–CH_3–	31.32
49	n-6 *trans*	ω3	–CH_2–CH_2–CH_3–	31.2
50	Caproic FA C6:0	ω3	–CH_2–CH_2–CH_3–	31.03
51	All FA		–$(CH_2)_n$–	29.56-28.73
52	Unsaturated FA		–CH_2–CH=CH–	26.91-26.88
53	PUFA		–CH=CH–CH_2–CH=CH–	25.34
54	All FA except butyric		–OOC–CH_2–CH_2–	24.58
55	All FA except caproic,caprylic, and n-3	ω2	–CH_2–CH3–	22.48
56	Caprylic FA C8:0	ω2	–CH_2–CH3–	22.4
57	Caprylic FA C6:0	ω2	–CH_2–CH3–	22.08
58	n-3	ω2	–CH_2–CH3–	20.13
59	Butyric FA	C3	–CH_2–CH3–	18.1
60	All FA	ω1	–CH3	13.85-13.79-13.62-13.33

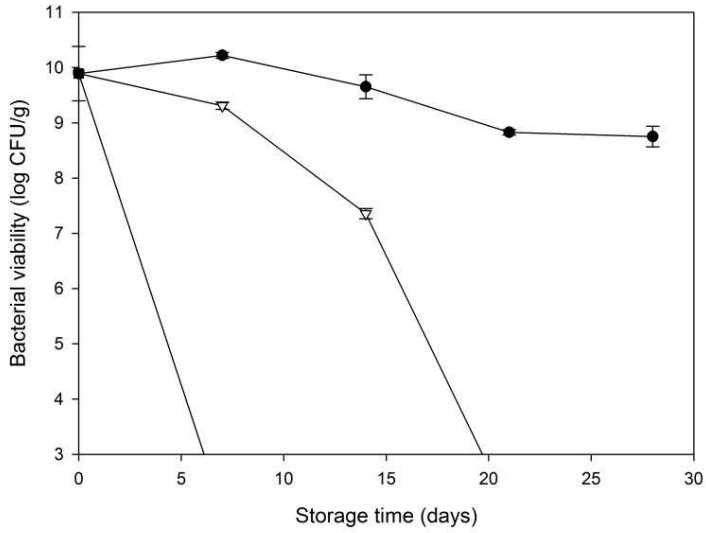


Figure A4 (a) The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in whole milk powder having 0.5 a_w. The viability is expressed as the logarithmic values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3)

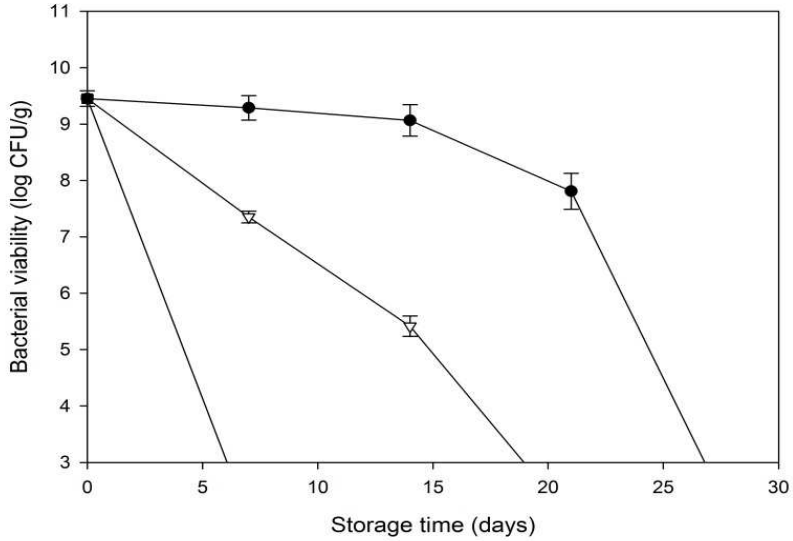


Figure A4 (b) The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in skim milk powder having 0.5 a_w. The viability is expressed as the logarithmic

values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3)

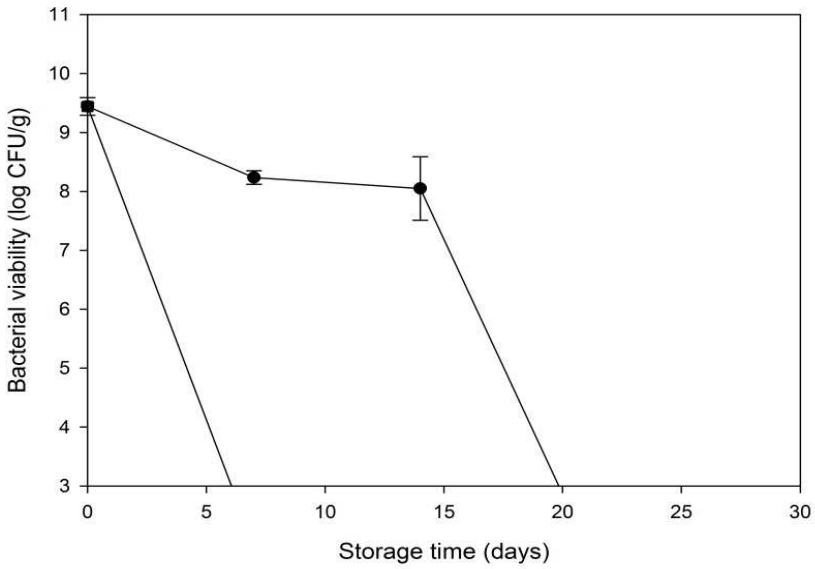


Figure A4 (b) The storage stability of fluidized bed dried *Lactobacillus paracasei* powders embedded in milk protein isolate having 0.5 a_w. The viability is expressed as the logarithmic values of survival against storage time of 4 weeks at 4 °C (black circle), 25 °C (white triangle), and 37 °C (black square). Error bars represent standard deviation of means (n≥3)

APPENDIX B:

Table B1 Dilution with Boron nitride required for transmission

Compound		Moles BN per mole com-pound	Mwt com-pound	**% com-pound by weight (in BN)**	muT d	Step	Sample thick-ness for pack-ing density of 1 mm
61 ppm Mn in matrix					1	0.002	1
105 ppm Mn in matrix					1	0.004	1
$MnCO_3.H_2O$	Mn C O4 H2	20000	114.9	**0.023**	0.92	0.004	1
$Mn(CH_3COO)_2.4H_2O$	Mn C4 O8 H14	25000	245.1	**0.039**	0.92	0.004	1
$MnCl_2.4H_2O$	Mn Cl2 O4 H8	25000	197.9	**0.032**	0.92	0.004	1
$MnSO_4.H_2O$	Mn S O5 H2	25000	169.0	**0.027**	0.92	0.004	1
$Mn(HPO_4)(H_2O)_3$	Mn P2 O7 H7	25000	204.9	**0.033**	0.92	0.004	1
$Mn_3(PO_4)_2.3H_2O$	Mn3 P2 O11 H6	70000	408.8	**0.024**	0.92	0.004	1
$MnPO_4H_2O$	Mn P O5 H2	20000	167.9	**0.034**	0.92	0.004	1
$Mn(acac)_3$	Mn C15 O6 H21	20000	355.3	**0.072**	0.92	0.004	1
Mn(II) acetate dihy-drate	Mn C6 O8 H13	20000	268.1	**0.054**	0.92	0.004	1
MnO_2		20000	86.9	**0.018**	0.92	0.004	1

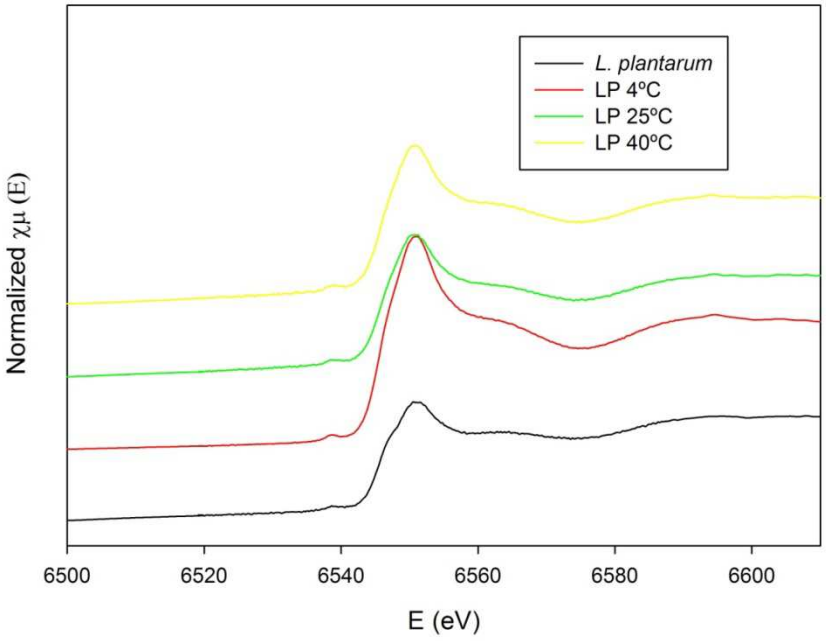


Figure B-1 XANES spectrum of Lactobacillus plantarum stored at 4ºC, 25ºC and 37ºC

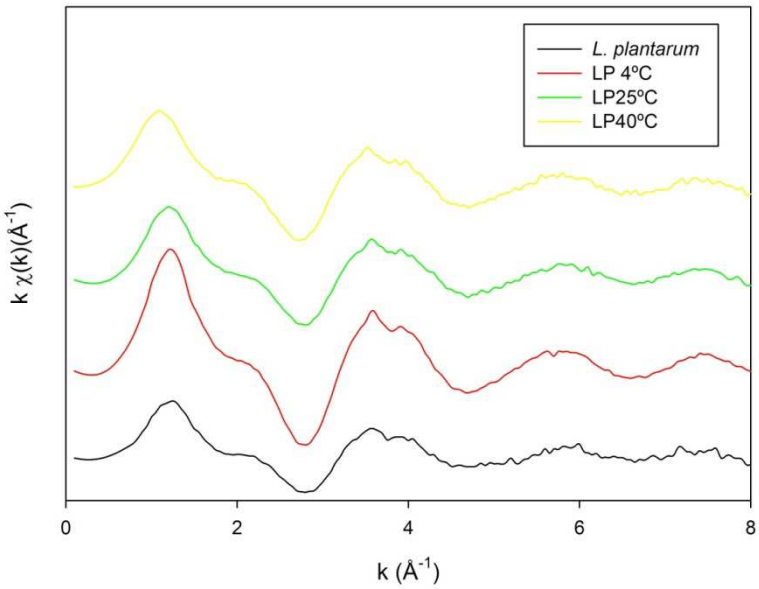


Figure B-2 EXAFS spectrum of *Lactobacillus plantarum* stored at 4ºC, 25ºC and 37ºC

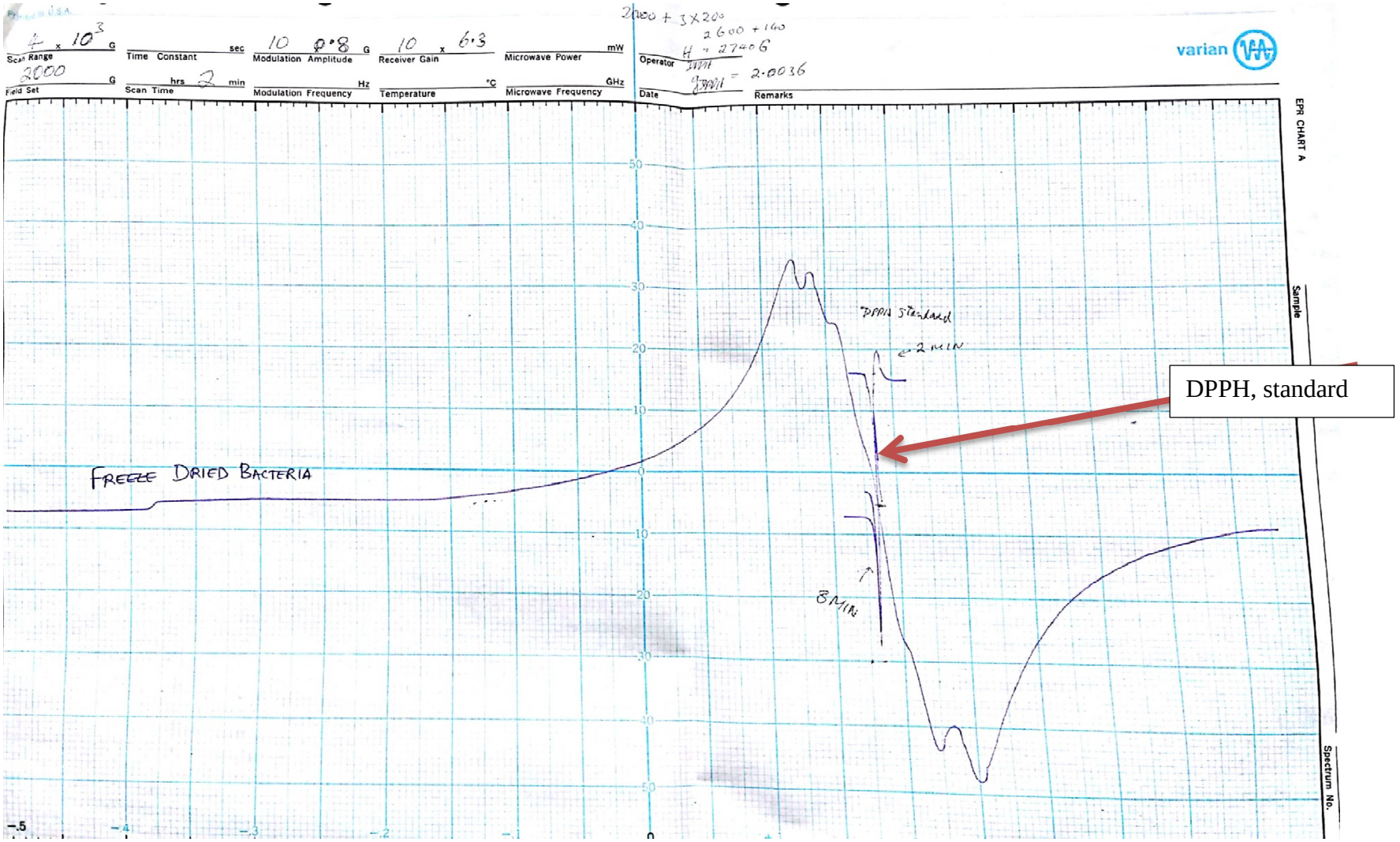


Fig B-3. ESR spectrum of Freeze dried bacteria in Dairy matrix, stored at 0.5 a_w and DPPH standard.

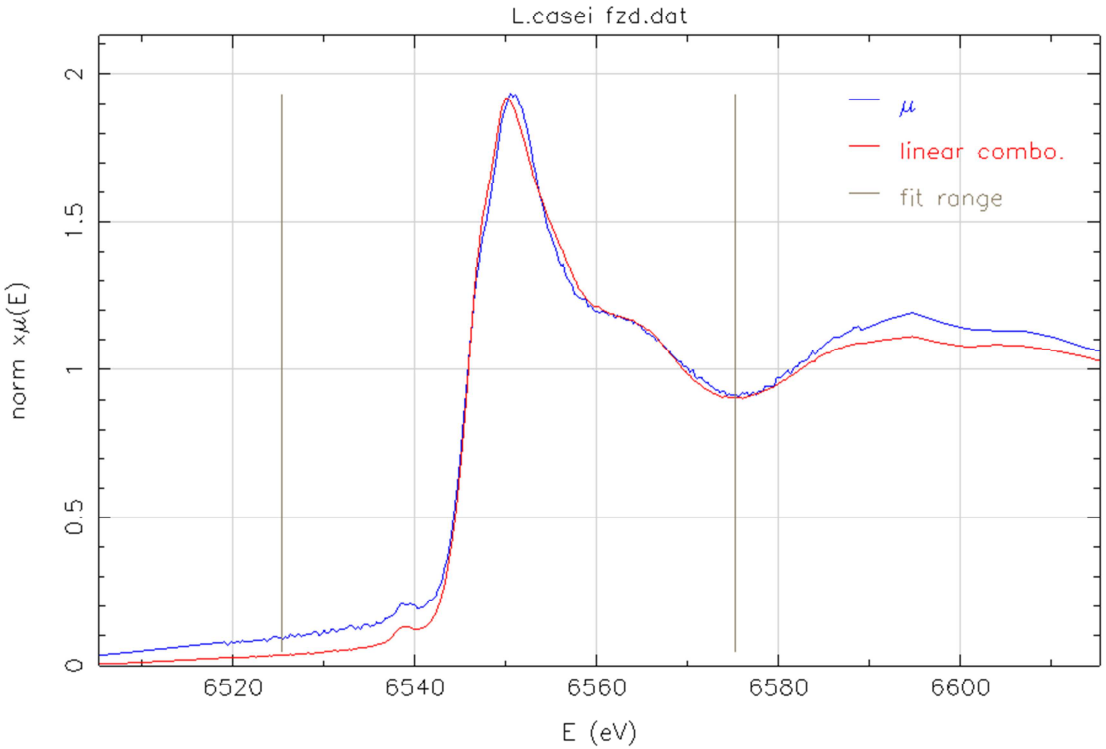
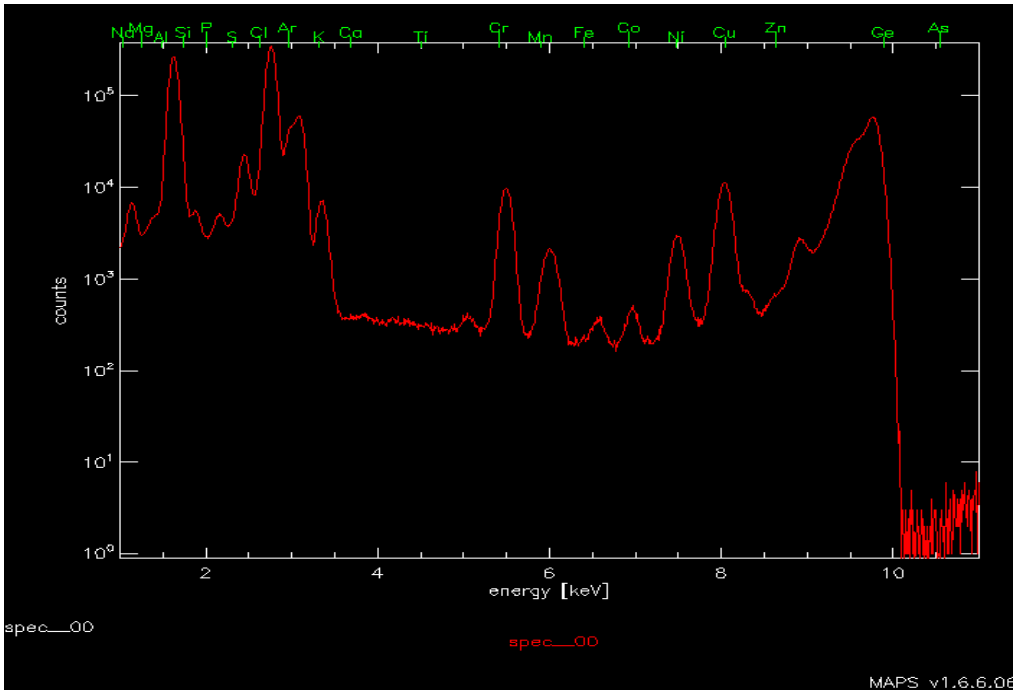
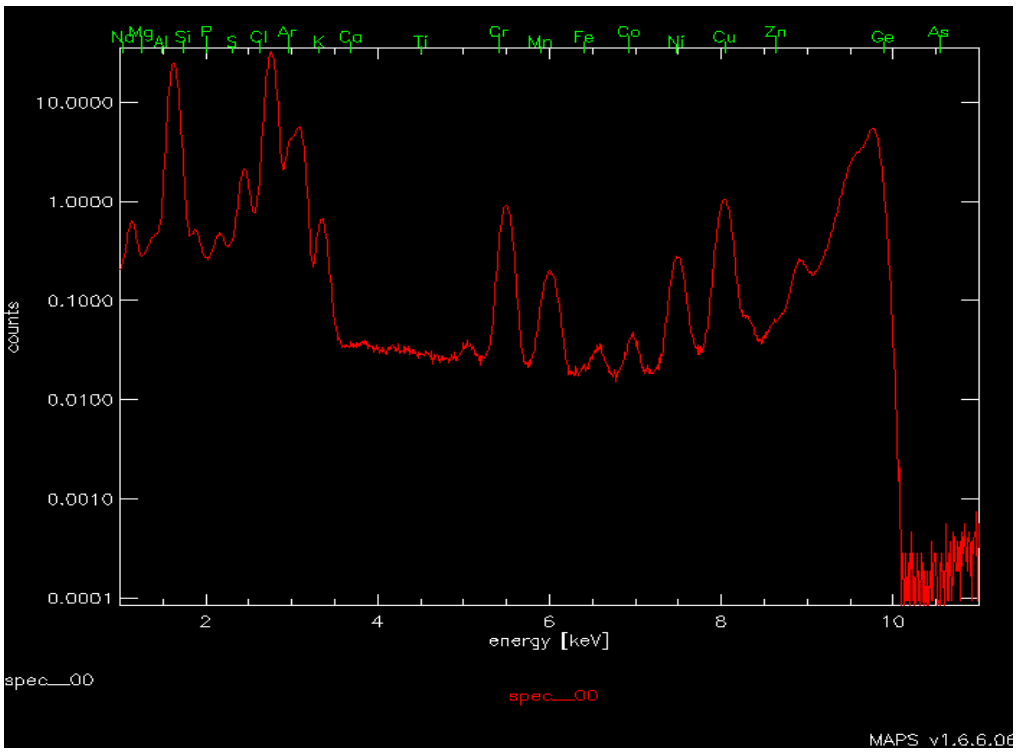
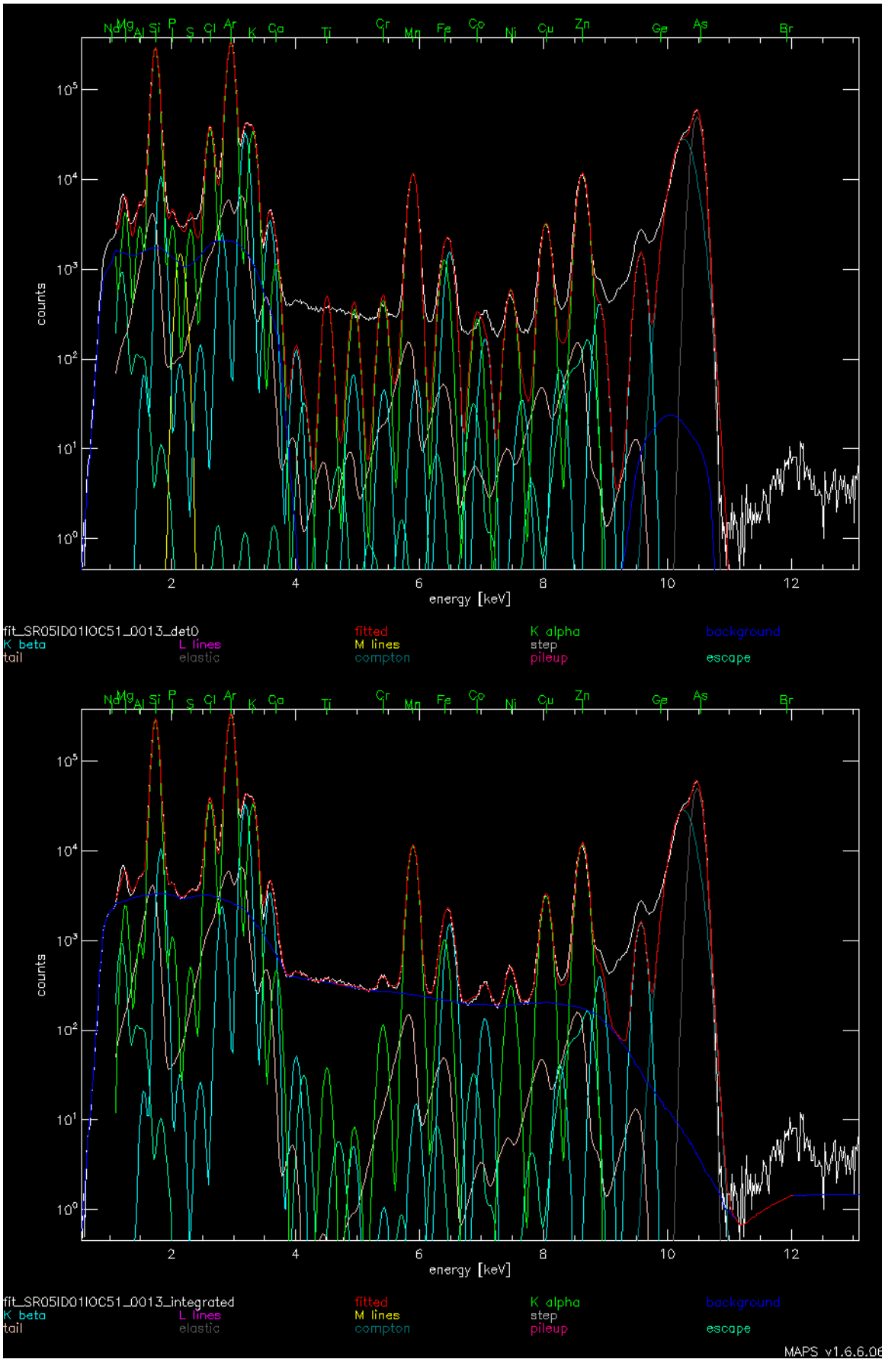


Fig B-4. XANES best fit of the XANES absorption spectrum of *Lactobacillus paracasei* to that of the model compound manganese phosphate and manganese acetate.

APPENDIX C: XAF calibration graphs for quantification of elements using known elemental standards



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energy [keV]
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fitted
K alpha
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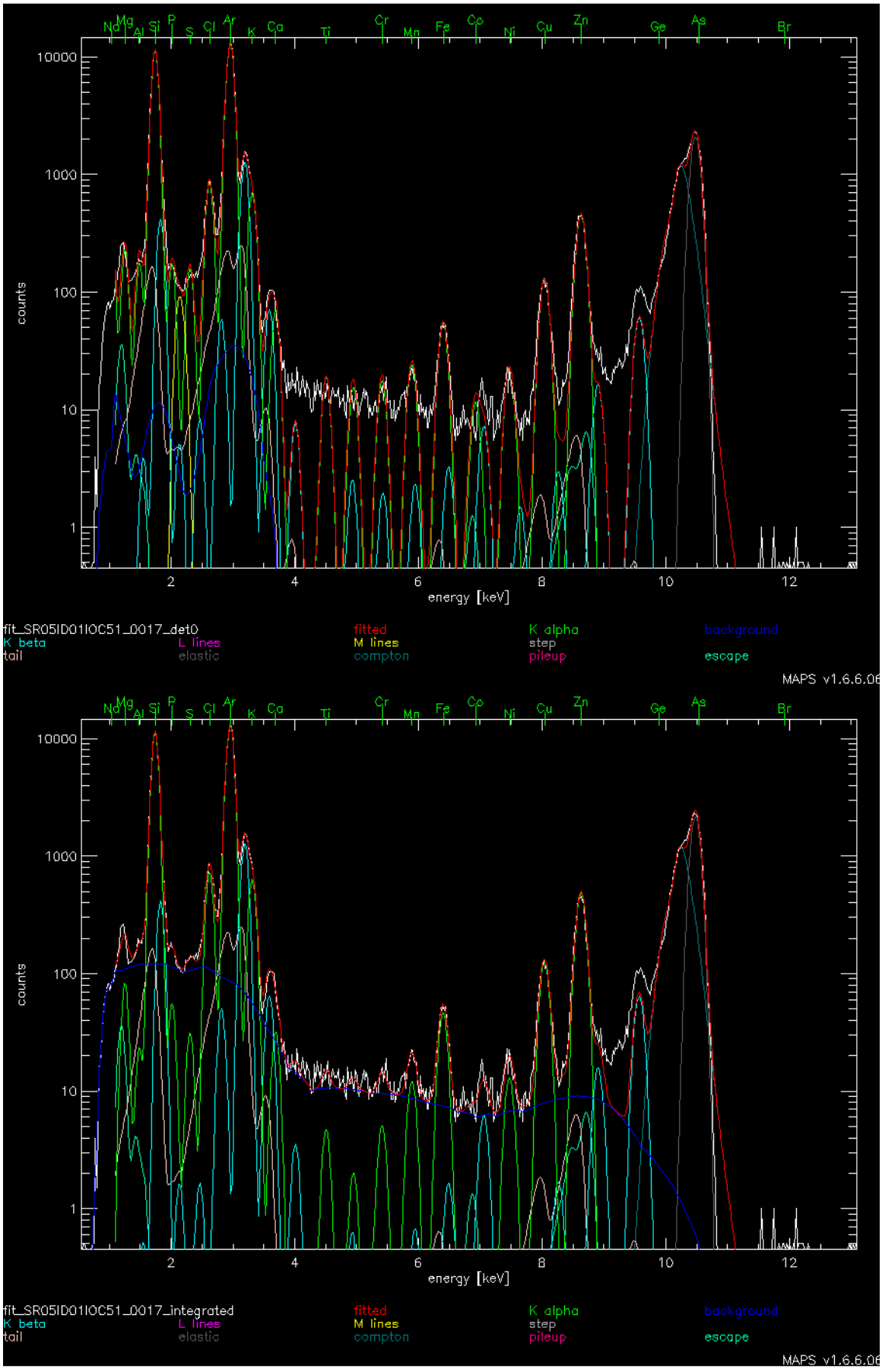
fit_SR05ID01IOC51_0013_integrated
MAPS v1.6.6.06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MAPS v1.6.6.06
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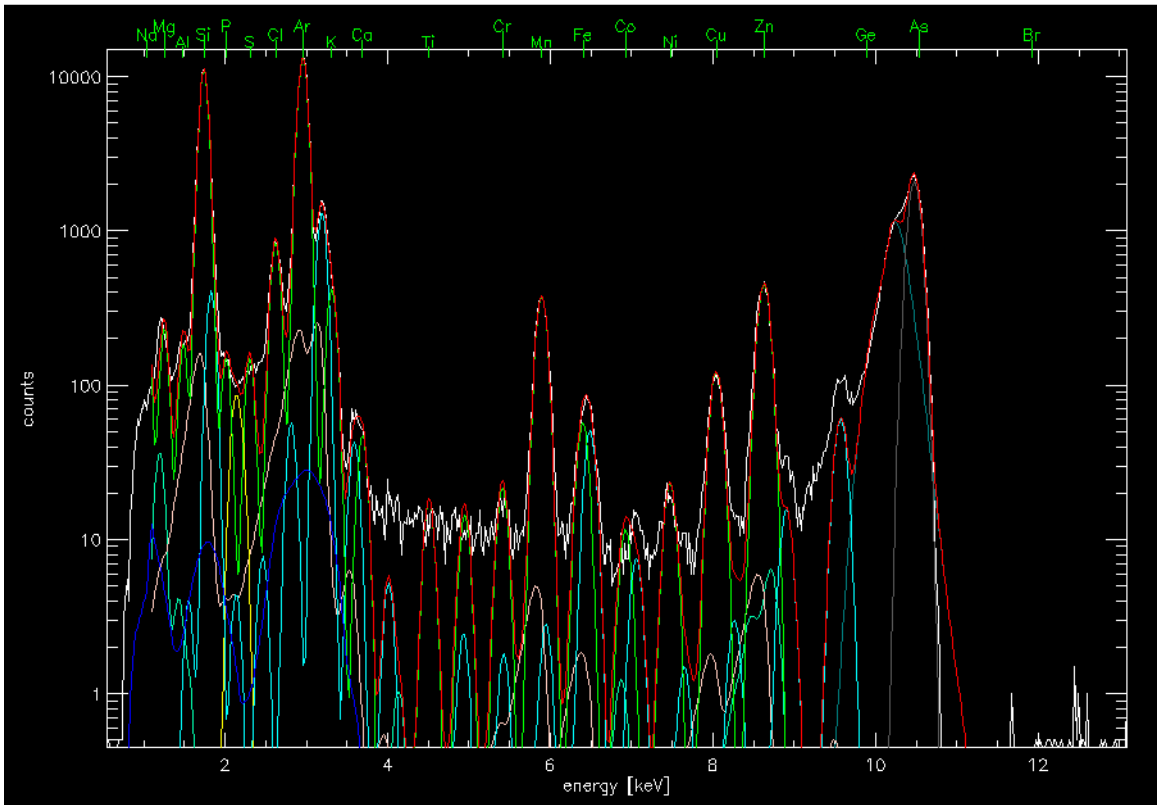
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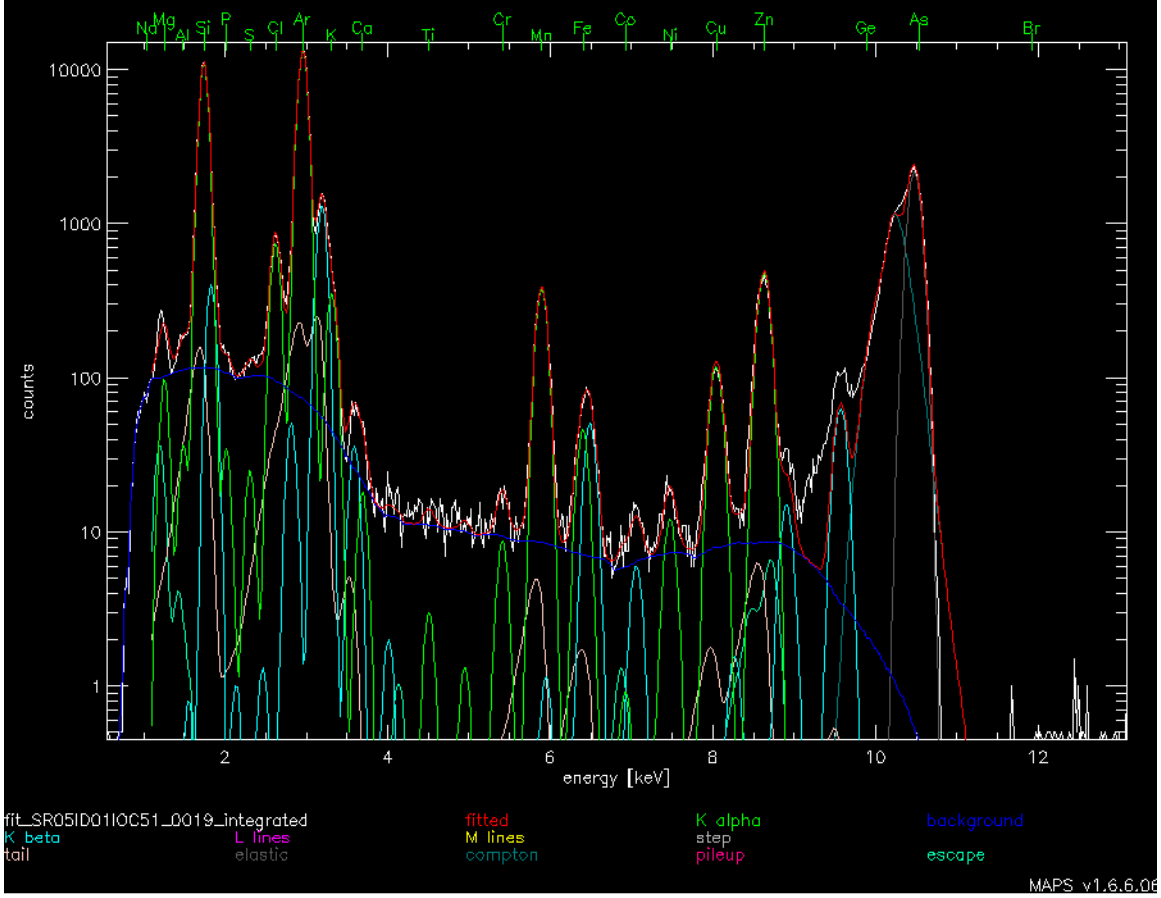
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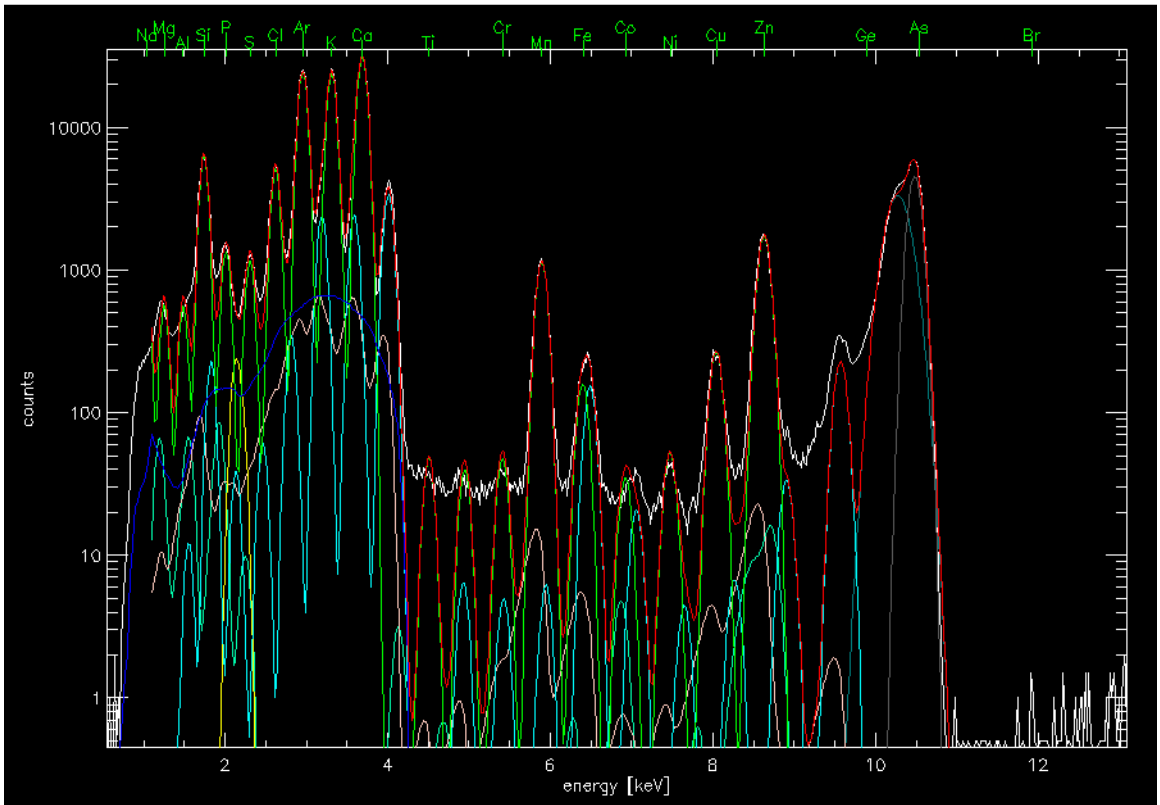
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counts
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energy [keV]
fit_SR05ID01IOC51_0023_det0
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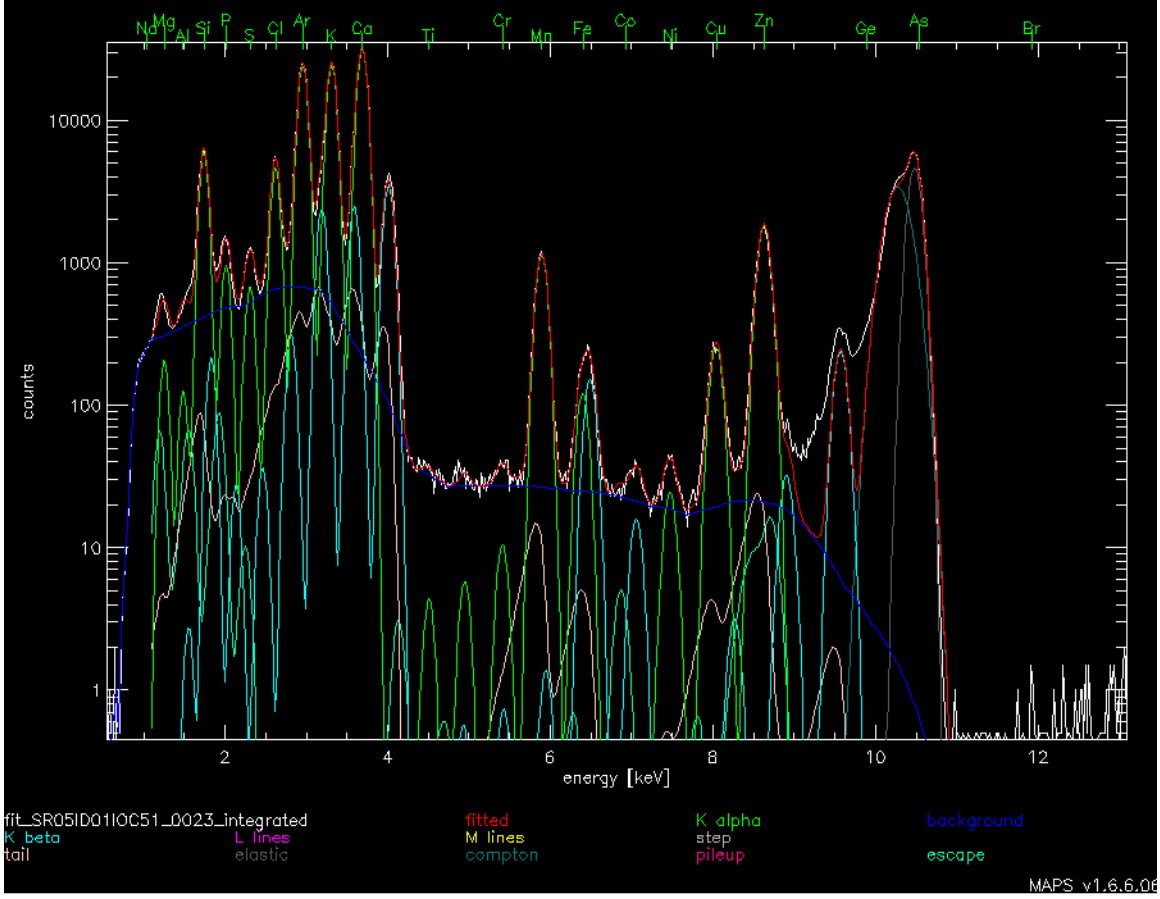
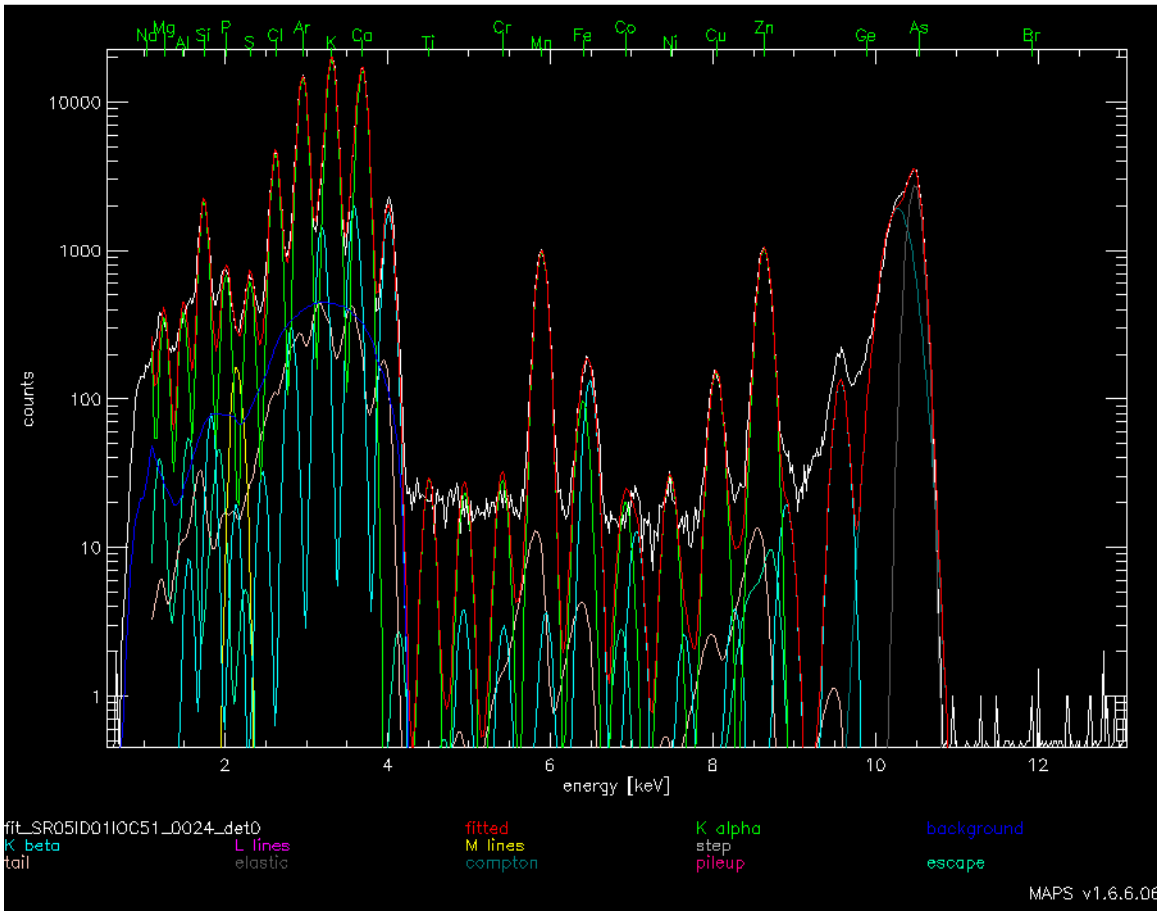
escape
MAPS v1.6.6.06


Na Mg Al Si P S Cl Ar K Ca Ti Cr Mn Fe Co Ni Cu Zn Ge As Br
10000
1000
100
10
1
counts
2
4
6
8
10
12
energy [keV]
fit_SR05ID01IOC51_0023_integrated
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06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10000
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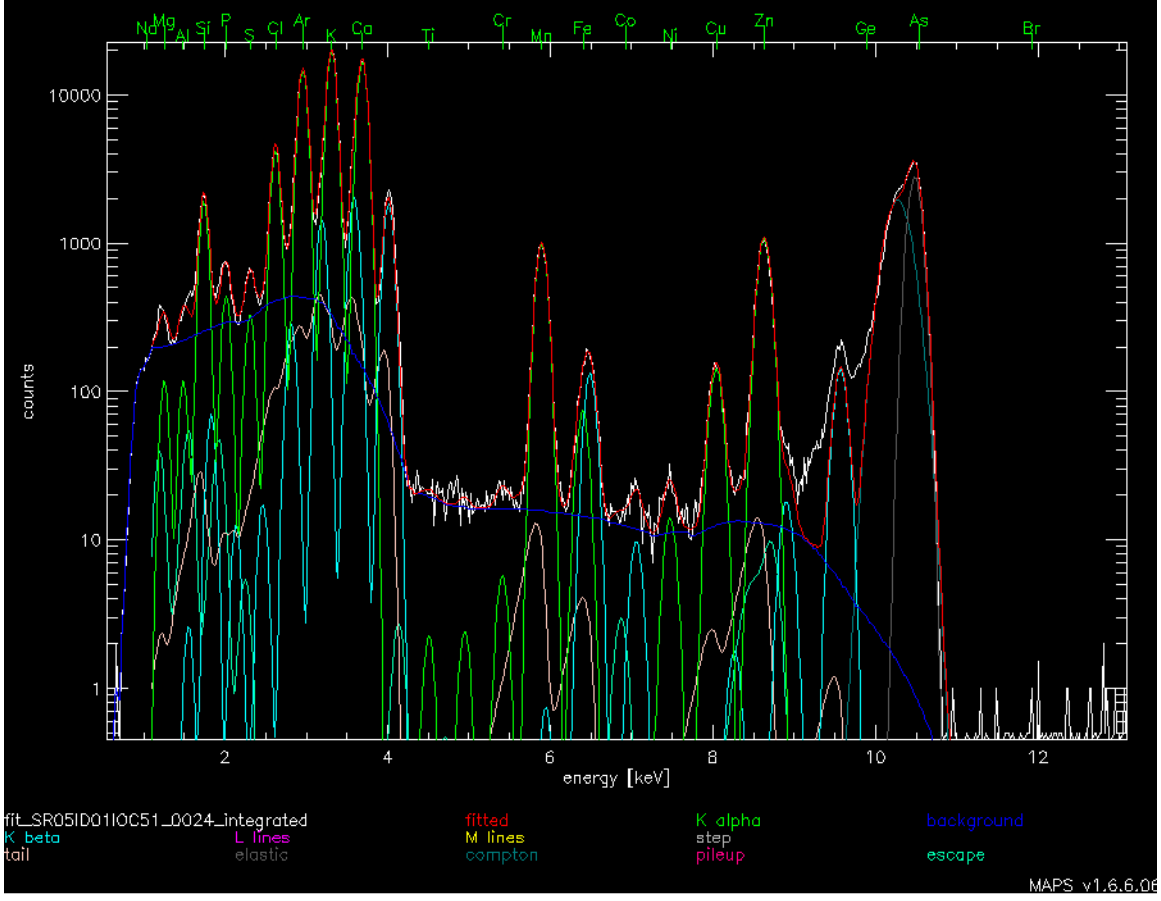
100
10
1
counts
2
4
6
8
10
12
energy [keV]
fit_SR05ID01IOC51_0024_det0
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06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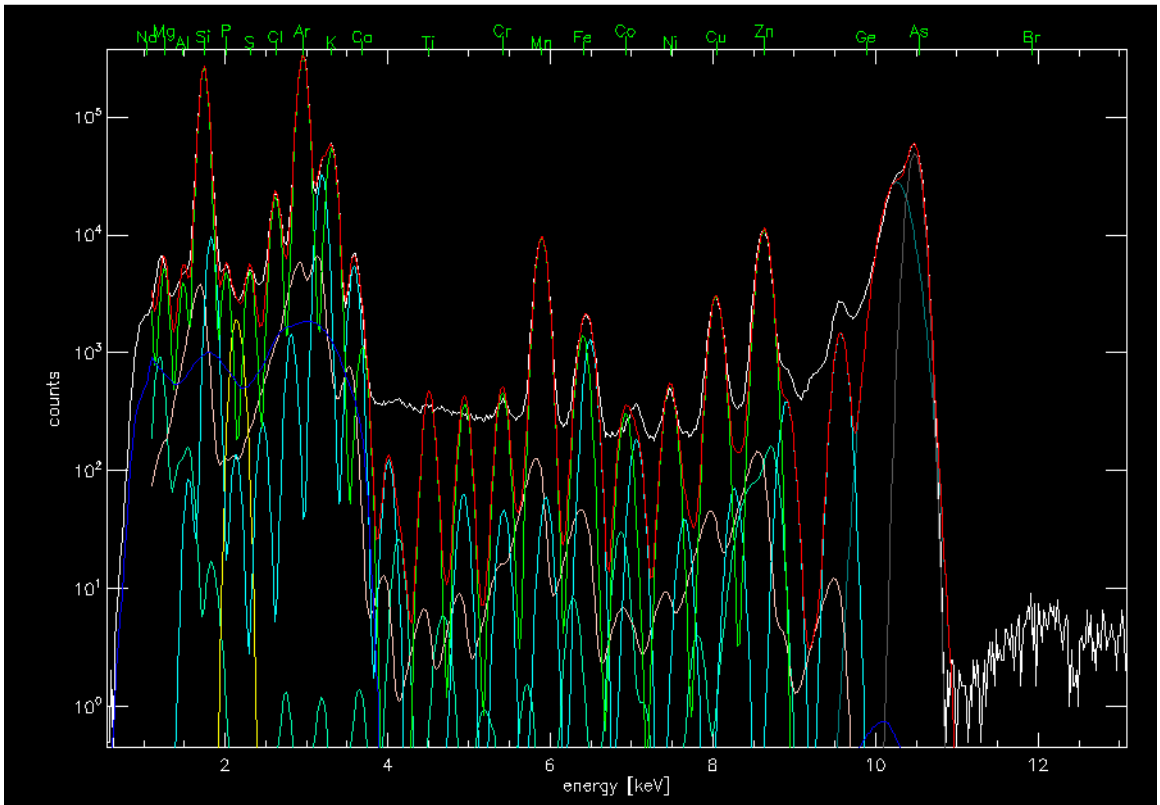
10000
1000
100
10
1
counts
2
4
6
8
10
12
energy [keV]
fit_SR05ID01IOC51_0024_integrated
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06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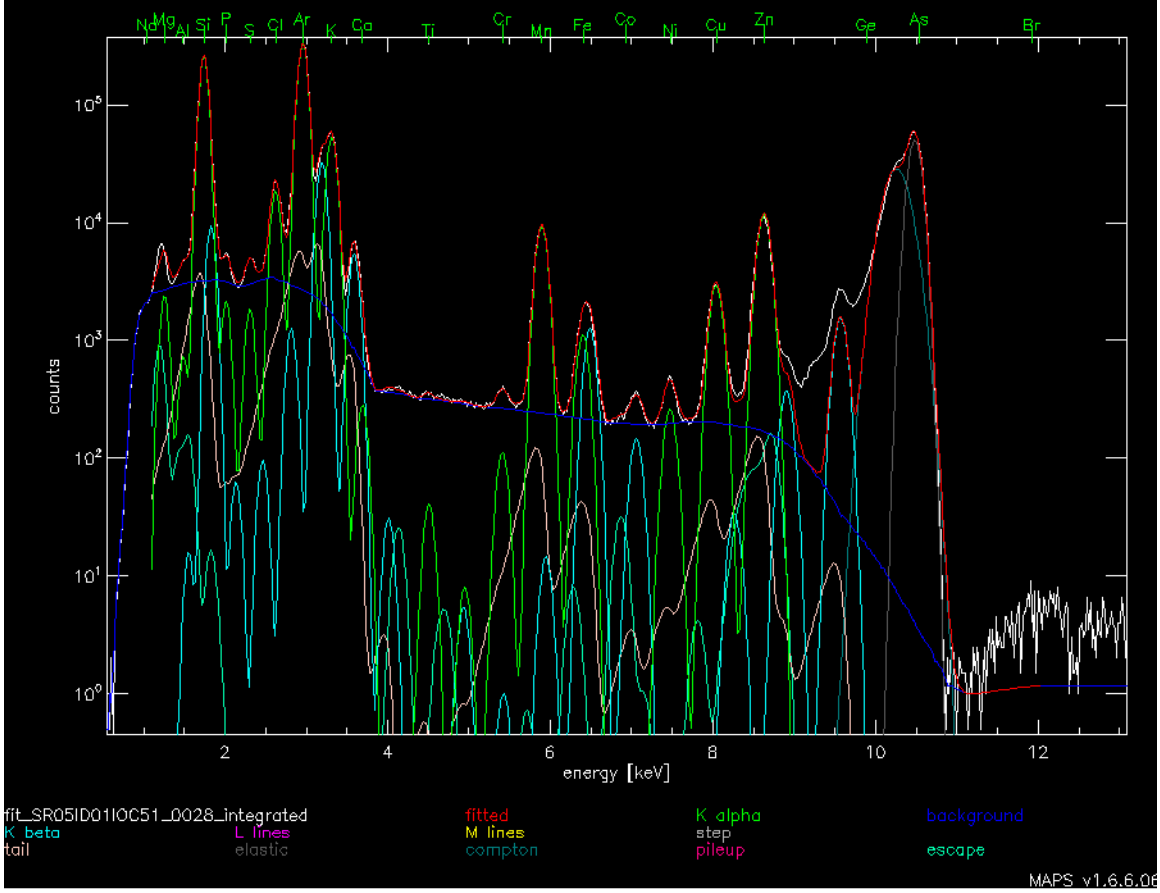
counts
energy [keV]
fit_SR05ID01IOC51_0028_det0
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06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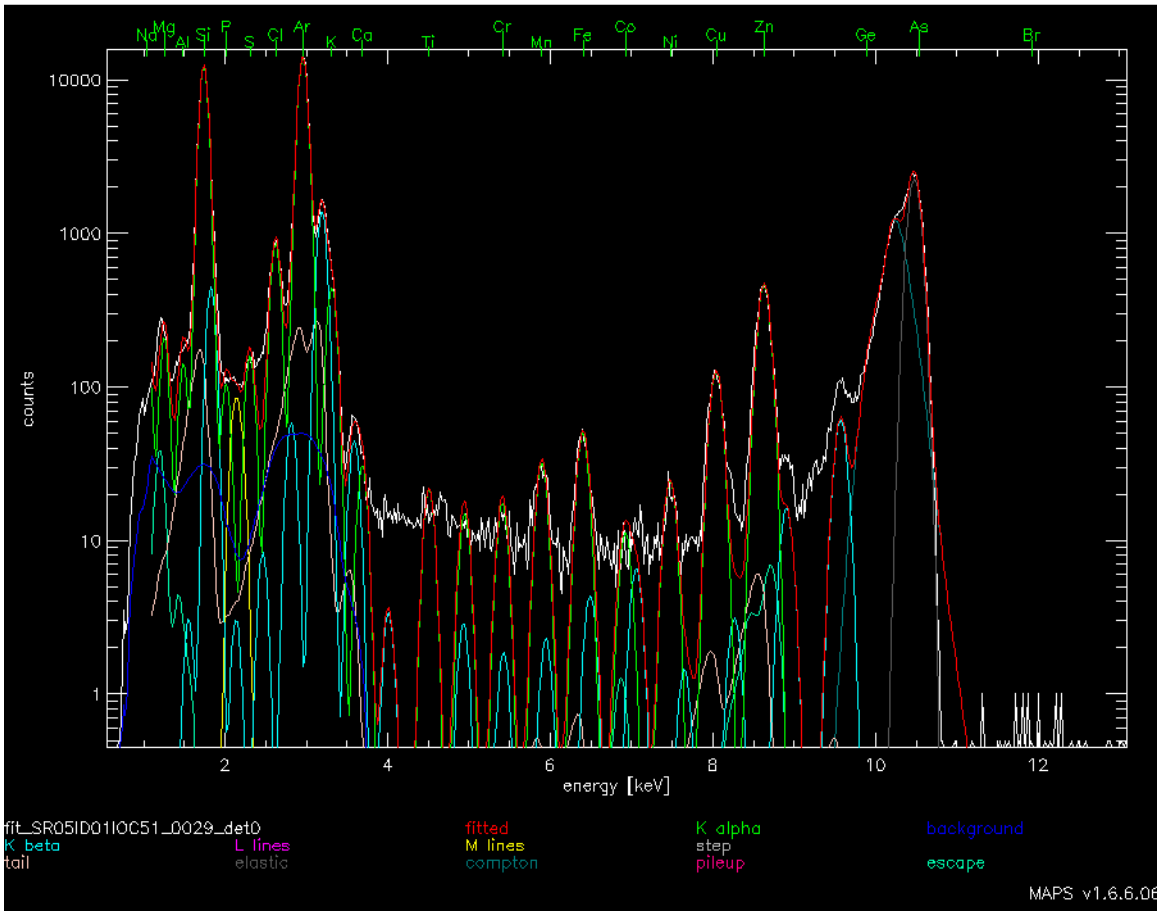
counts
energy [keV]
fit_SR05ID01IOC51_0028_integrated
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06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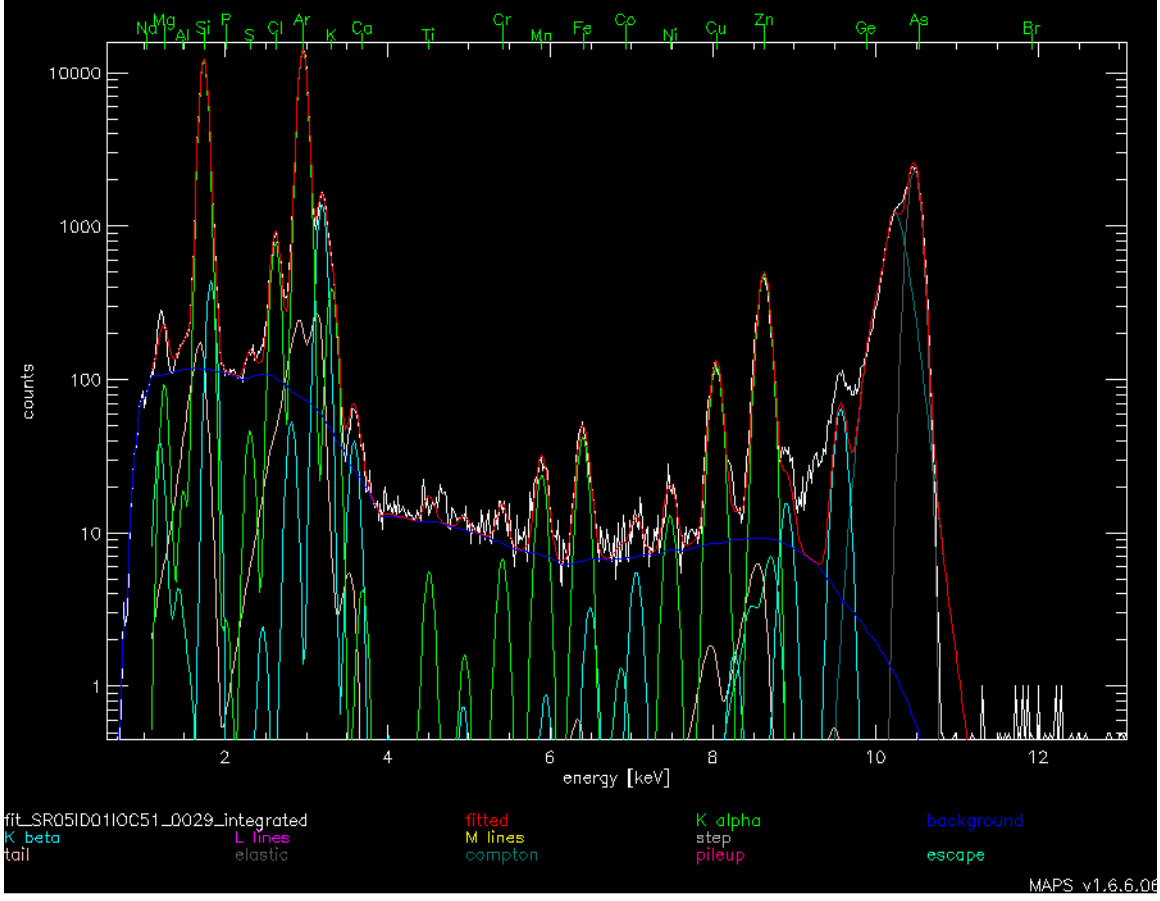
10000
1000
100
10
1
counts
2
4
6
8
10
12
energy [keV]
fit_SR05ID01IOC51_0029_det0
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06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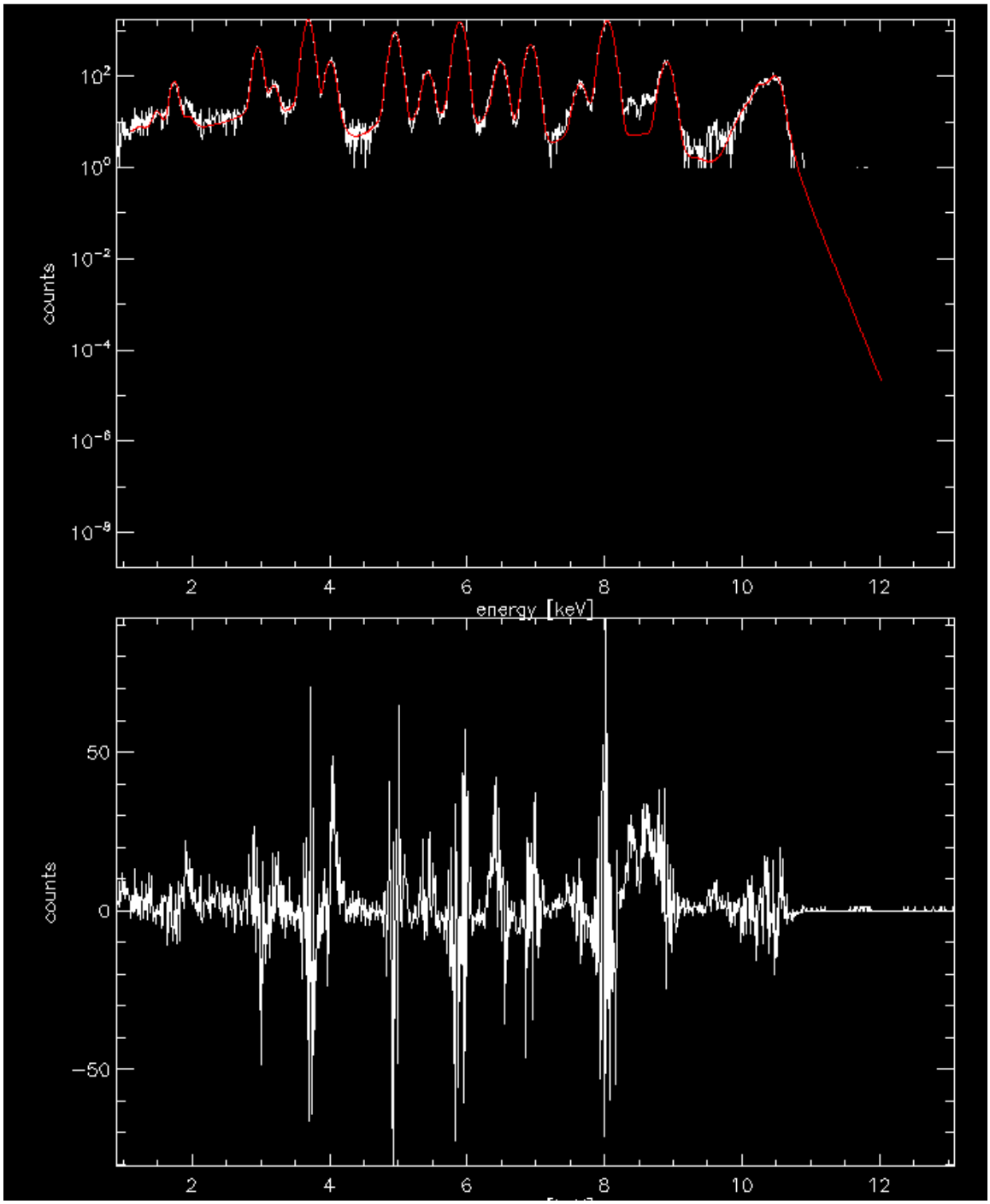
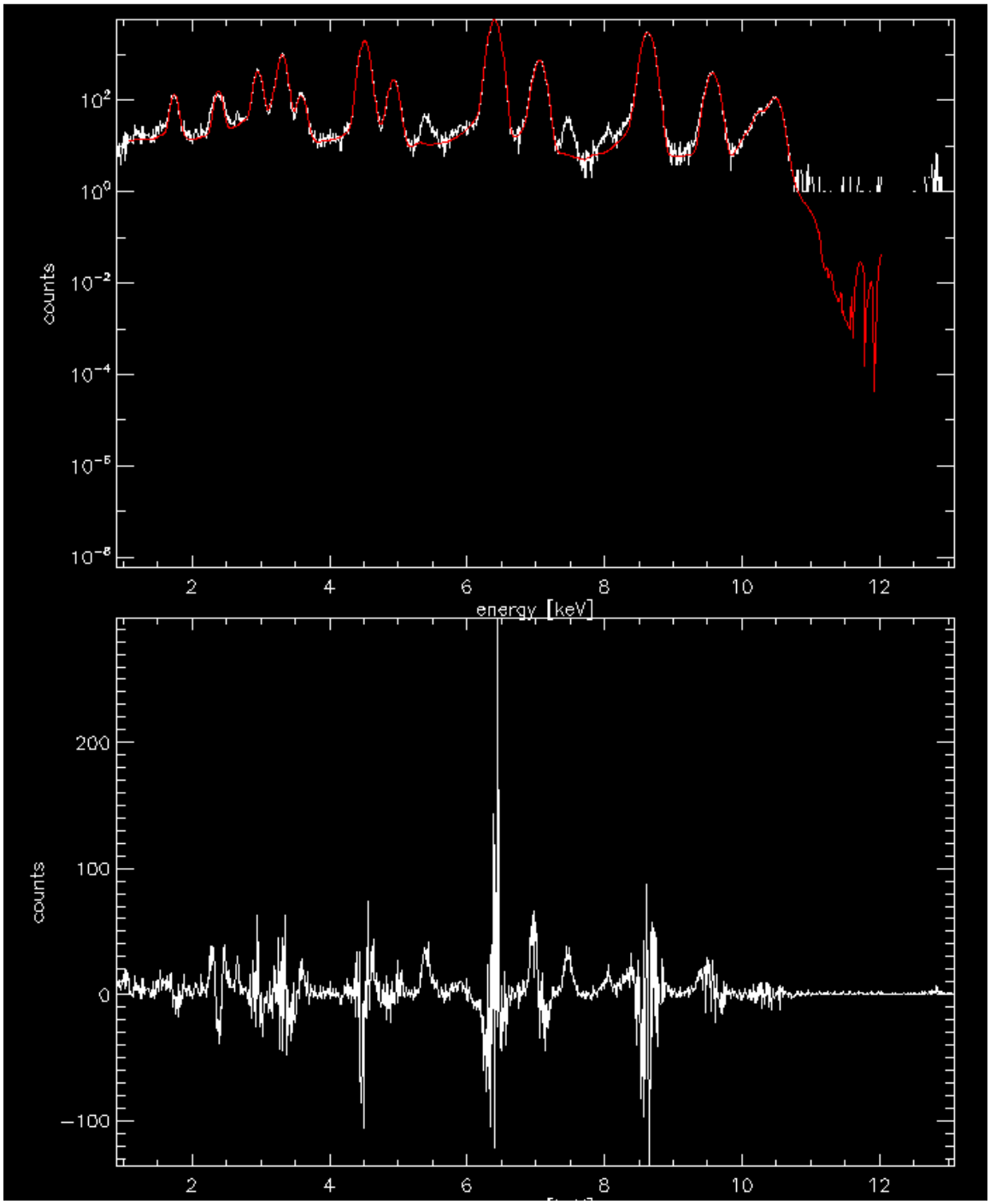
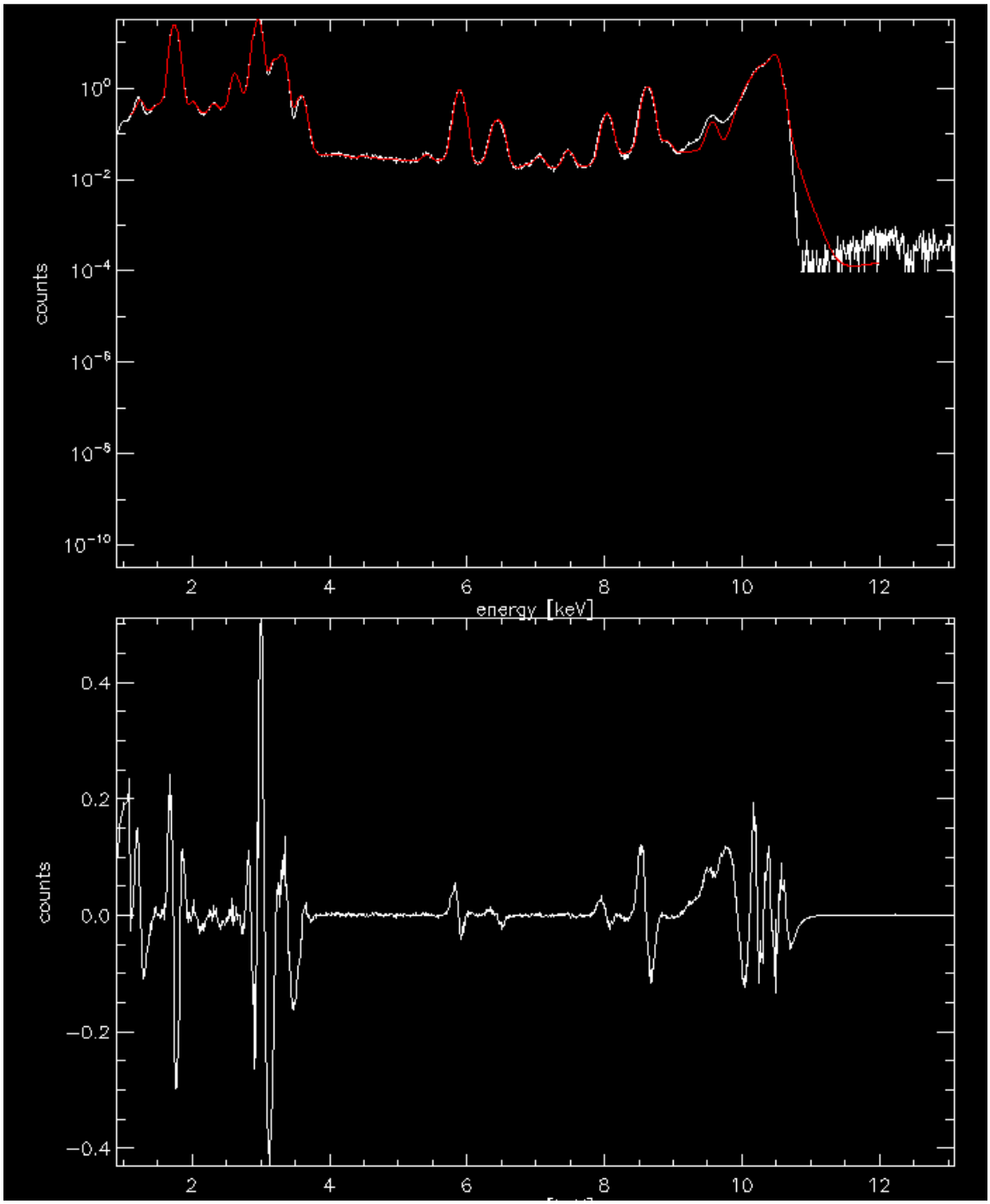
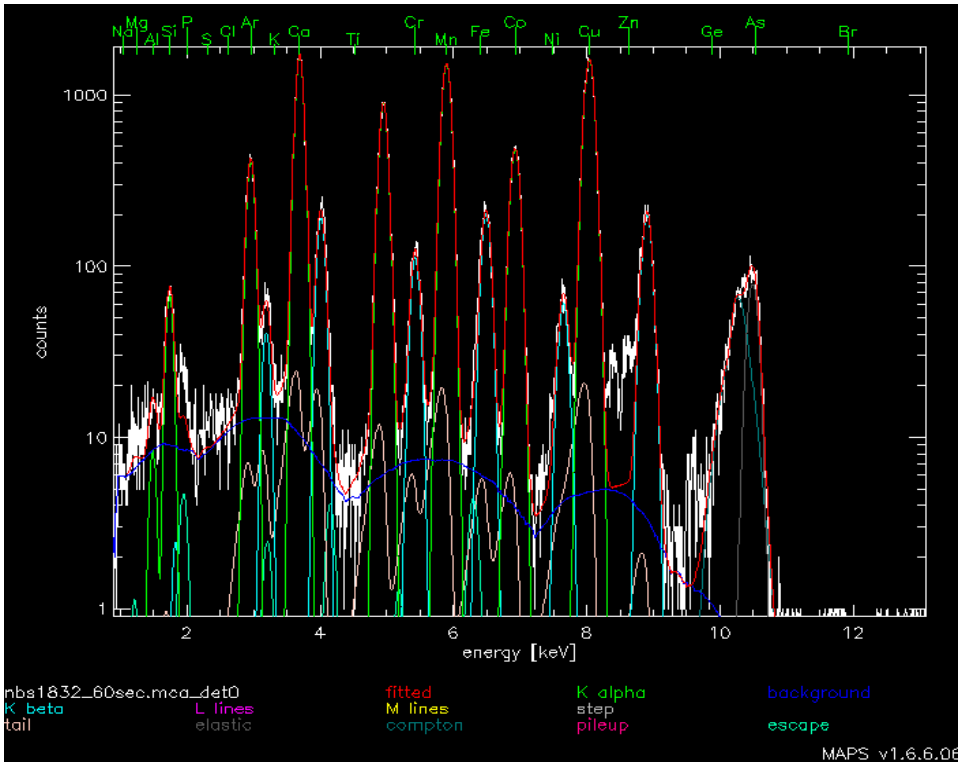
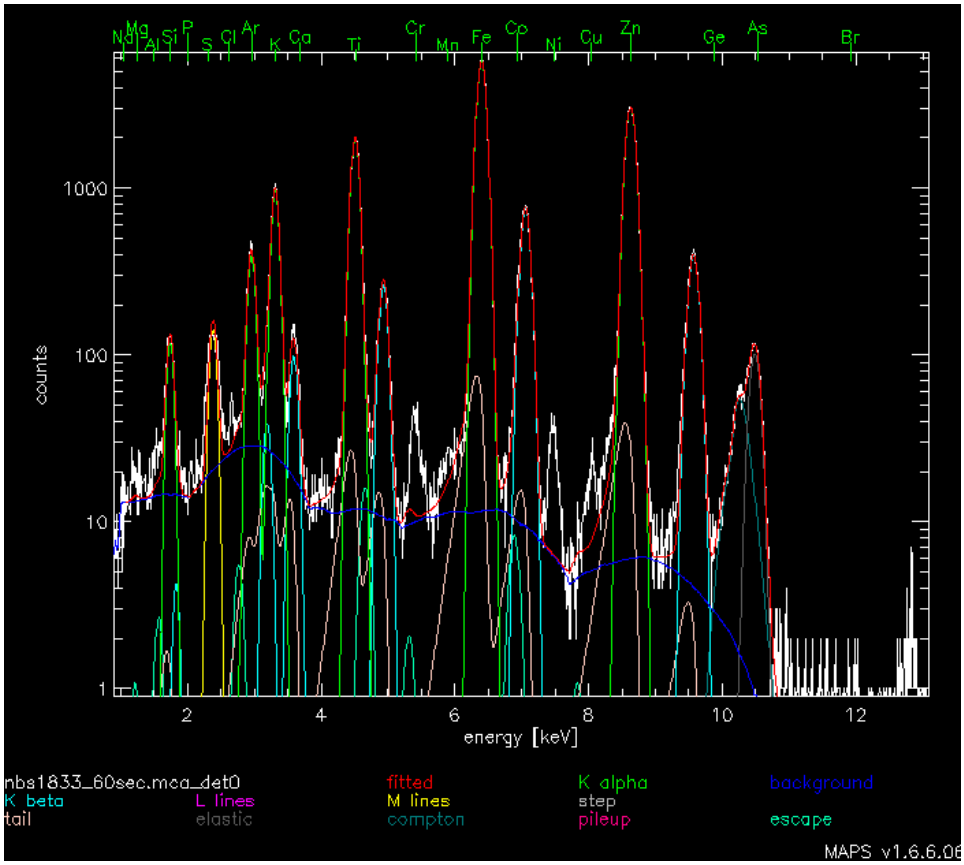
10000
1000
100
10
1
counts
2
4
6
8
10
12
energy [keV]
fit_SR05ID01IOC51_0029_integrated
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06

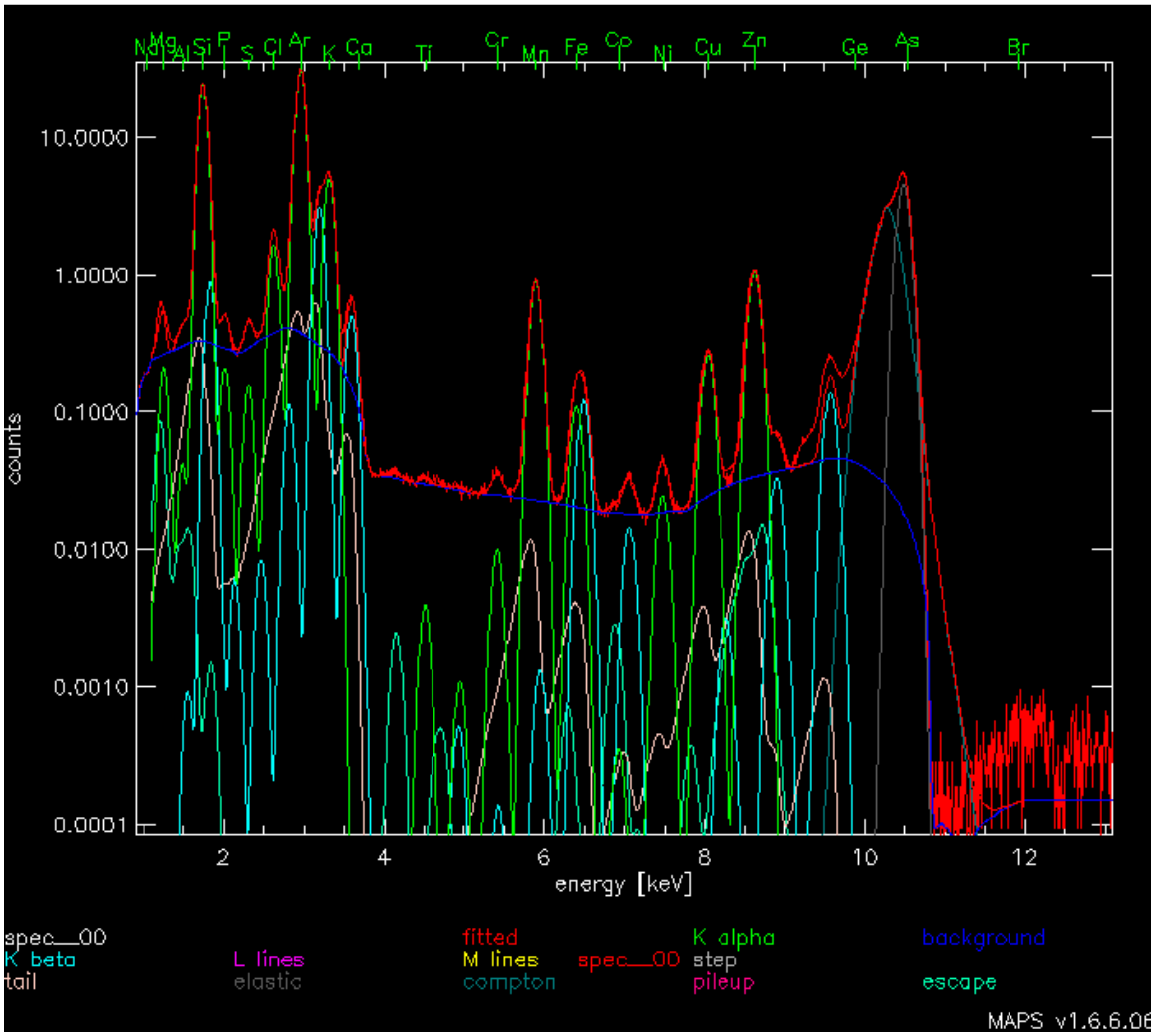

counts
energy [keV]
counts


counts
energy [keV]
counts


counts
energy [keV]
counts


Na Mg Al Si P S Cl Ar K Ca Ti Cr Mn Fe Co Ni Cu Zn Ge As Br
1000
100
10
1
counts
2
4
6
8
10
12
energy [keV]
nbs1832_60sec.mca_det0
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06


Na Mg Al Si P S Cl Ar K Ca Ti Cr Mn Fe Co Ni Cu Zn Ge As Br
1000
100
10
1
counts
2
4
6
8
10
12
energy [keV]
nbs1833_60sec.mca_det0
fitted
K alpha
background
K beta
L lines
M lines
step
tail
elastic
compton
pileup
escape
MAPS v1.6.6.06


Na Mg Al Si P S Cl Ar K Ca Ti Cr Mn Fe Co Ni Cu Zn Ge As Br
10.0000
1.0000
0.1000
0.0100
0.0010
0.0001
counts
2
4
6
8
10
12
energy [keV]
spec__00
K beta
tail
L lines
elastic
fitted
M lines
compton
spec__00
K alpha
step
pileup
background
escape
MAPS v1.6.6.06